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⑤4 2'-5'-Phosphorothioat-Oligoadenylate und deren antivirale Verwendungen

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(54) 2',5'-Phosphorothioate oligoadenylates and antiviral uses thereof

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(56) References cited:
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- **NUCLEOSIDES AND NUCLEOTIDES**, vol. 6, no. 1,2, 26 May 1987, pages 173-184, XP002002974 **K.KARIKO ET AL.**: "Phosphorothioate Analogs of 2-5A: Elucidation of the Stereochemical Course of the Enzymes of the 2-5A Synthetase / RNase L System."
- **BIOCHEMISTRY**, vol. 24, no. 3, 29 January 1985, EASTON, PA US, pages 551-555, XP002002975 **C.LEE ET AL.**: "2',5'-Oligoadenylates Chiral at Phosphorus : Enzymatic Synthesis, Properties and Biological Activities of 2',5'-Phosphorothioate Trimer and Tetramer Analogues Synthesized from (Sp)-ATPalphas."
- **BIOCHEMISTRY**, vol. 26, no. 22, November 1987, EASTON, PA US, pages 7127-7135, XP002002976 **K.KARIKO ET AL.**: "Phosphorothioate Analogues of 2',5'-Oligoadenylate. Enzymatically Synthesized 2',5'-Phosphorothioate Dimer and Trimer: Unequivocal Structural Assignment and Activation of 2',5'-Oligoadenylate-Dependent Endoribonuclease."
- **BIOCHEMISTRY**, vol. 26, no. 11, November 1987, EASTON, PA US, pages 7136-7142, XP002002977 **K.KARIKO ET AL.**: "Phosphorothioate Analogues of 2',5'-Oligoadenylate. Activation of 2',5'-Oligoadenylate-Dependent Endoribonuclease by 2',5'-Phosphorothioate Cores and 5'-Monophosphates."
- **Science** Vol. 216, pp 1415-1416(1982)

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Description**Field of the Invention**

5 [0001] The invention relates to synthetic analogues of naturally occurring antiviral 2',5'-oligoadenylylates wherein the internucleotide phosphodiester linkages are replaced with optically active phosphorothioate groups. The compounds have increased metabolic stability where the stereoconfiguration around one or more of the chiral phosphorous atoms is the Sp configuration.

10 **Reference to Government Grant**

[0002] The invention described herein was made, in part, in the course of work supported by National Institutes of Health grant POI CA-29545 and National Science Foundation grant DMB84-15002.

15 **Background of the Invention**

[0003] The full nomenclature of the subject matter of the present invention involves extremely long terms. It is customary for those skilled in the art to abbreviate oligoadenylylate analogues and related terms in a manner well-known to them. These general and customary abbreviations are set forth herein below and may be utilized in the text of this specification.

Abbreviations:

[0004]

25 2-5A, 2',5'-oligoadenylylate or p_3A_n : Oligomer of adenylic acid with 2',5'-phosphodiester linkages and a 5'-terminal triphosphate group.

A_2 , A_3 and A_4 : Dimer, trimer and tetramer of adenylic acid with 2',5'-phosphodiester linkages.

pA_3 , ppA_3 (or p_2A_3), $pppA_3$ (or p_3A_3): 5'-terminal mono-, di- and triphosphates of A_3 .

30 AMPS: Adenosine 5'-Q-phosphorothioate.

SVPD: Snake venom phosphodiesterase.

2'-PDE: 2'-phosphodiesterase

Rp: The R stereoconfiguration about a chiral phosphorous atom in a phosphorothioate internucleotide linkage.

Sp: The S stereoconfiguration about a chiral phosphorous atom in a phosphorothioate internucleotide linkage.

35 RNase L: 2-5A dependent endoribonuclease.

$A_{Rp}A_{Rp}A$: (Rp)-P-thioadenylyl-(2'-5')-(Rp)-P-thioadenylyl-(2'-5')-adenosine.

$A_{Sp}A_{Rp}A$: (Sp)-P-thioadenylyl-(2'-5')-(Rp)-P-thioadenylyl-(2'-5')-adenosine.

$A_{Rp}A_{Sp}A$: (Rp)-P-thioadenylyl-(2'-5')-(Sp)-P-thioadenylyl-(2'-5')-adenosine.

$A_{Sp}A_{Sp}A$: (Sp)-P-thioadenylyl-(2'-5')-(Sp)-P-thioadenylyl-(2'-5')-adenosine.

40 $pA_{Rp}A_{Rp}A$, $ppA_{Rp}A_{Rp}A$, $pppA_{Rp}A_{Rp}A$, $pA_{Sp}A_{Rp}A$, $ppA_{Sp}A_{Rp}A$, $pppA_{Sp}A_{Rp}A$, $pA_{Rp}A_{Sp}A$, $ppA_{Rp}A_{Sp}A$, $pppA_{Rp}A_{Sp}A$, $pA_{Sp}A_{Sp}A$, $ppA_{Sp}A_{Sp}A$, and $pppA_{Sp}A_{Sp}A$: 5'-mono-, di- and triphosphates of $A_{Rp}A_{Rp}A$, $A_{Sp}A_{Rp}A$, $A_{Rp}A_{Sp}A$ and $A_{Sp}A_{Sp}A$.

$A_{Rp}A_{Rp}A_{Rp}A$: (Rp)-P-thioadenylyl-(2'-5')-(Rp)-P-thioadenylyl-(2'-5')-(Rp)-P-thioadenylyl-(2'-5')-adenosine.

$A_{Rp}A_{Sp}A_{Rp}A$: (Rp)-P-thioadenylyl-(2'-5')-(Sp)-P-thioadenylyl-(2'-5')-(Rp)-P-thioadenylyl-(2'-5')-adenosine.

45 $A_{Rp}A_{Rp}A_{Sp}A$: (Rp)-P-thioadenylyl-(2'-5')-(Rp)-P-thioadenylyl-(2'-5')-(Sp)-P-thioadenylyl-(2'-5')-adenosine.

$A_{Rp}A_{Sp}A_{Sp}A$: (Rp)-P-thioadenylyl-(2'-5')-(Sp)-P-thioadenylyl-(2'-5')-(Sp)-P-thioadenylyl-(2'-5')-adenosine.

$A_{Sp}A_{Rp}A_{Rp}A$: (Sp)-P-thioadenylyl-(2'-5')-(Rp)-P-thioadenylyl-(2'-5')-(Rp)-P-thioadenylyl-(2'-5')-adenosine.

$A_{Sp}A_{Sp}A_{Rp}A$: (Sp)-P-thioadenylyl-(2'-5')-(Sp)-P-thioadenylyl-(2'-5')-(Rp)-P-thioadenylyl-(2'-5')-adenosine.

$A_{Sp}A_{Rp}A_{Sp}A$: (Sp)-P-thioadenylyl-(2'-5')-(Rp)-P-thioadenylyl-(2'-5')-(Sp)-P-thioadenylyl-(2'-5')-adenosine.

50 $A_{Sp}A_{Sp}A_{Sp}A$: (Sp)-P-thioadenylyl-(2'-5')-(Sp)-P-thioadenylyl-(2'-5')-(Sp)-P-thioadenylyl-(2'-5')-adenosine.

$pA_{Rp}A_{Rp}A_{Rp}A$, $ppA_{Rp}A_{Rp}A_{Rp}A$, $pppA_{Rp}A_{Rp}A_{Rp}A$, $pA_{Rp}A_{Sp}A_{Rp}A$, $ppA_{Rp}A_{Sp}A_{Rp}A$, $pppA_{Rp}A_{Sp}A_{Rp}A$, $pA_{Rp}A_{Rp}A_{Sp}A$, $ppA_{Rp}A_{Rp}A_{Sp}A$, $pppA_{Rp}A_{Rp}A_{Sp}A$, $pA_{Rp}A_{Sp}A_{Sp}A$, $ppA_{Rp}A_{Sp}A_{Sp}A$, $pppA_{Rp}A_{Sp}A_{Sp}A$, $pA_{Sp}A_{Rp}A_{Rp}A$, $ppA_{Sp}A_{Rp}A_{Rp}A$, $pppA_{Sp}A_{Rp}A_{Rp}A$, $pA_{Sp}A_{Sp}A_{Rp}A$, $ppA_{Sp}A_{Sp}A_{Rp}A$, $pppA_{Sp}A_{Sp}A_{Rp}A$, $pA_{Sp}A_{Rp}A_{Sp}A$, $ppA_{Sp}A_{Rp}A_{Sp}A$, $pppA_{Sp}A_{Rp}A_{Sp}A$, $pA_{Sp}A_{Sp}A_{Sp}A$, $ppA_{Sp}A_{Sp}A_{Sp}A$, $pppA_{Sp}A_{Sp}A_{Sp}A$: 5'-mono-, di- and triphosphates of the above tetramers.

(Sp) - ATP-alpha-S: Adenosine 5'-Q-(Sp)-(1-thiotriphosphate).

Background of the Invention

[0005] The 2-5A system is widely expected to be involved in the antiviral mechanism of interferon and may also be involved in the regulation of cell growth and differentiation. 2-5A synthesized from ATP by 2',5'-oligoadenylate synthetase [ATP: (2'-5')oligo(A)-adenyltransferase (EC 2.7.7.19)] exerts its biological effects by binding to and activating its only known target enzyme, the unique 2-5A-dependent endoribonuclease RNase L (EC 3.1.27). RNase L cleaves viral and cellular mRNA or rRNA, thereby inhibiting protein synthesis. Hovanessian et al, *Eur. J. Biochem.* **93**:515-526 (1979); Kerr et al, *Proc. Natl. Acad. Sci. USA.* **75**:256-260 (1978). It has been reported that 2-5A protects plant tissue from infection by tobacco mosaic virus. Devash et al, *Science* **216**:415 (1982). 2-5A, however, is metabolically unstable. It is degraded by a cellular 2'-phosphodiesterase and phosphatases. Knight et al, *Meth. Enzymol.* **79**:216-227 (1981); Minks et al, *Nucleic Acids Res.* **6**:767-780 (1979); Williams et al, *Eur. J. Biochem.* **92**:455-462 (1978).

[0006] The literature is replete with structurally-modified 2-5A molecules with modifications in the adenylyl or ribosyl moiety designed to explore the biological role of the 2-5A synthetase/RNase L system. The primary source of conformational flexibility in the 2-5A molecule is in the backbone, similar to 3',5'-linked RNA and DNA. Srinivasan et al, *Nucleic Acids Res.* **13**:5707-5716 (1985). However, theoretical and experimental analyses have revealed that the conformation of 2', 5'-linked dinucleotides and polynucleotide chains are significantly different from 3',5'-linked nucleotides. Id. The ribose-phosphate backbone of 2-5A has also been demonstrated to be the major antigenic determinant in the molecule. Johnston et al, *Biochemistry* **22**:3453-3460 (1983).

[0007] Few reports have appeared on the synthesis of 2-5A analogues with backbone modifications. Core analogues containing methylphosphonate and methylphosphotriester groups have been synthesized. Eppstein et al, *J. Biol. Chem.* **257**:13390-13397 (1982); Jager et al, *Nucleic Acids Res. Sym. Ser. No. 9*:149-152 (1981). However, complete loss of activity was observed with the "uncharged" methylphosphotriester analogues. Eppstein et al, *supra*. Substitution of the 2',5'-phosphodiester linkages with 3', 5'-linkages has also lead to substantial decrease in biological activity. Lesiak et al, *J. Biol. Chem.* **258**:13082-13088 (1983). Replacement of only one 2',5'-internucleotide linkage has resulted in at least one order of magnitude loss of activity. Nearly complete loss of biological activity was observed when both 2',5'-phosphodiester linkages in the 2-5A trimer were replaced with 3',5'-bonds.

[0008] Haugh et al, *Eur. J. Biochem.* **132**:77-84 (1983), reported that the affinity of pA₃ to RNase L in mouse L929 cell extracts is approximately 1,000 times greater than that of A₃.

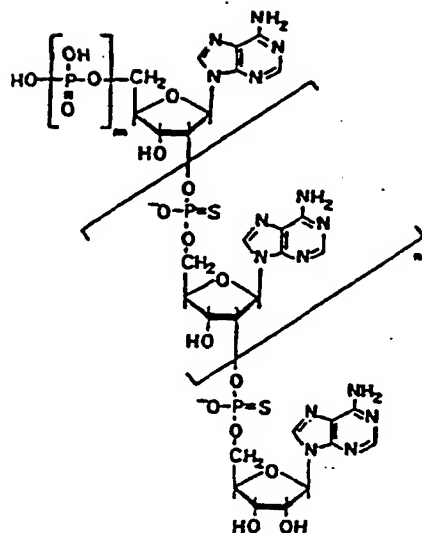
[0009] Nelson et al, *J. Org. Chem.* **49**:2314-2317 (1984), describe diastereomeric pairs of the phosphorothioate analogue of A₃ without resolution of individual enantiomers. Eppstein et al, *J. Biol. Chem.* **261**:5999-6003 (1986) report metabolic stabilities and antiviral activity of purported A_{Rp}A_{Rp}A/A_{Sp}A_{Rp}A and A_{Rp}A_{Sp}A/A_{Sp}A_{Sp}A racemic mixtures without resolution of individual enantiomers.

[0010] Lee and Suhadolnik, *Biochemistry* **24**:551-555 (1985), and Suhadolnik and Lee in *The 2-5A System: Molecular and Clinical Aspects of the Interferon-Regulator Pathway*, Williams, B.R.G. and Silverman, R.H., Eds. (1985), Alan R. Liss, Inc., New York, p. 115-122, disclose the enzymatic synthesis of the alpha-phosphorothioate 5'-triphosphates of A_{Rp}A_{Rp}A and A_{Rp}A_{Rp}A_{Rp}A from (Sp)-ATP-alpha-S. Such compounds are metabolically unstable. Preparation of the corresponding stereoisomers with Sp internucleotide phosphorothioate linkages was not possible owing to the stereospecificity of 2-5A synthetase for the substrate (Sp)-ATP-alpha-S, which is inverted to yield trimer and tetramer products containing 2',5'-phosphorothioate internucleotide linkages of the Rp configuration exclusively. Because nucleoside transferases provide exclusively the inverted configuration when Sp-ATP-alpha-S is the substrate, 2',5'-phosphorothioate oligoadenylates containing internucleotide phosphorothioate groups of the Sp configuration cannot be synthesized enzymatically.

Summary of the Invention

[0011] Compounds of the present invention useful in inhibiting viral infections in plants and mammals have increased metabolic stability and/or antiviral activity.

[0012] In accordance with the present invention there are provided compounds and water-soluble salts thereof of the formula I

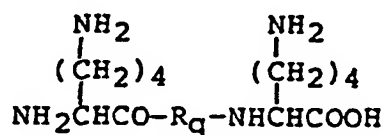


I

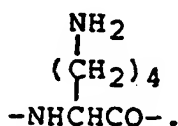
wherein m is zero, 1, 2 or 3 and n is 2.

[0013] The present invention also provides a method of controlling viral infection in plants in accordance with claim 1.

[0014] In another embodiment of the present invention compounds according to the formula I wherein n is 2 are utilized to form oligoadenylate conjugates with the macromolecular carrier poly(L-lysine) for intracellular transport. Such poly(L-lysine)/2',5'-phosphorothioate oligoadenylate conjugates have the formula



wherein q is an integer from 60 to 70, and R is randomly R' or



From five to ten of the R groups comprise R'. R' has the following formula wherein m is 0, 1, 2 or 3:



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Detailed Description of the Invention

[0017] The activation of the unique 2-5A-dependent endoribonuclease, RNase L, by 2',5'-oligoadenylates, and its hydrolysis of rRNA, tRNA and cellular and viral mRNA, is important in the inhibition of viral replication and regulation of cell growth. RNase L is known to be the substrate target for 2-5A. It is one of the chief functional enzymes of the interferon-induced biological cascade. While modified analogues of 2-5A have been reported, they are not metabolically stable, or fail to activate RNase L. The introduction of the phosphorothioate group in the 2',5' -internucleotide linkages of 2-5A, induces physical, chemical and biochemical modifications in the 2-5A molecule including (i) *Rp/Sp* chirality, (ii) lowered pKa, (iii) altered metal ion chelation, (iv) charge modulation, (v) increased bond length, (vi) altered degree of hydration and (vii) increased metabolic stabilities.

[0018] The metabolic stability of the 2',5'-phosphorothioate oligoadenylates is greater than authentic 2-5A. This metabolic stability is greatly enhanced where at least one of the internucleotide phosphorothioate 2',5'-linkages is of the *Sp* configuration. While racemic mixtures of the trimer cores have been reported, Nelson et al, *J. Org. Chem.* 49:2314-2317 (1984) and Eppstein et al, *J. Biol. Chem* 261:5999-6003 (1986), efforts to resolve the compounds have failed. The level of antiviral activity of, the trimer core racemates reported by Eppstein et al is such that the dosages required for treatment would be prohibitively toxic. At least one of the purported racemates of Eppstein et al, the *A_{Rp}A_{Sp}A/A_{Sp}A_{Sp}A* racemate, is of little value since, as we have found, the *A_{Sp}A_{Sp}A* stereoisomer selectively inactivates RNase L; thereby preventing *A_{Rp}A_{Sp}A* from exerting its antiviral effect through activation of RNase L. This is most undesirable since, as we have found, *A_{Rp}A_{Sp}A* is the most attractive of the four trimer core stereoisomers, since it both activates RNase L and is metabolically stable.

[0019] We have succeeded in preparing the fully resolved 2',5'-phosphorothioate adenylate trimer cores, thus making possible the practical use of the important *A_{Rp}A_{Sp}A* stereoisomer. Our method of stereo-specific chemical synthesis also makes possible the preparation of the eight separate stereo isomers of the 2',5'-phosphorothioate tetramer. Preparation of the tetramer molecules enables conjugation with the carrier (poly)L-lysine, shown to be an effective vector for introducing 2',5'-oligoadenylates and analogues into intact cells. Poly(L-lysine) conjugation to trimer molecules is not feasible, owing to the destruction of the 2'-terminal ribosyl moiety and subsequent inactivation of the molecule. Conjugation to poly(L-lysine) permits efficient intracellular transport of the 2',5'-phosphorothioate oligoadenylates while preserving intact within the conjugate the trimer moiety believed necessary for good biological activity.

[0020] Correlation of biological properties with absolute configuration has only been possible with the preparation of the fully resolved 2',5'-phosphorothioate adenylate trimer cores described herein. However, the trimer core compounds have been found to bind and/or activate RNase L only modestly. We have found that the RNase L activation by the 2',5'-phosphorothioate core molecules is significantly enhanced by 5'-phosphorylation.

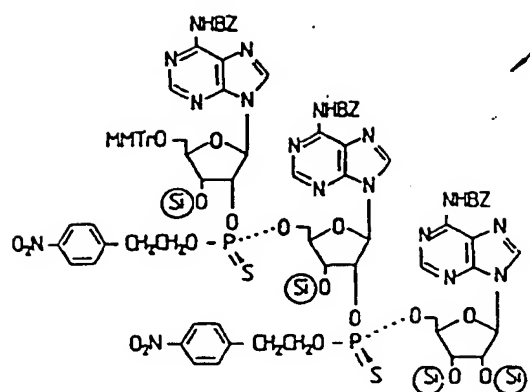
[0021] RNase L activation by authentic 2-5A requires the triphosphate form of the trimer. The 5'-monophosphate form of 2-5A is a potent inhibitor of the RNase L-activating activity of the triphosphate. Miyamoto et al., *J. Biol. Chem* 258: 15232-15237 (1983); Black et al., *FEBS Let.* 191:154-158 (1985); Torrence et al., *Proc. Natl. Acad. Sci. USA* 78:5993-5997 (1981). We have surprisingly found that the cores and monophosphates of the present phosphorothioate analogues of 2-5A, unlike authentic 2-5A, activate RNase L.

[0022] The phosphorothioate trimer cores *A_{Rp}A_{Rp}A*, *A_{Sp}A_{Rp}A*, *A_{Rp}A_{Sp}A*, and *A_{Sp}A_{Sp}A*, are chemically synthesized and separated by preparative thin layer chromatography on silica gel. The four trimer cores are prepared from 6-N-benzoyl-3'-O-*tert*-butyldimethylsilyl-5'-O-monomethoxytrityl-adenosine-2-O-(p-nitrophenylethyl)-octahydroazono-phosphoramidite by stereospecific synthesis, which relies on separation of fully resolved protected intermediates followed by removal of all blocking groups to yield the fully-resolved 2',5'-phosphorothioate trimer adenylate cores.

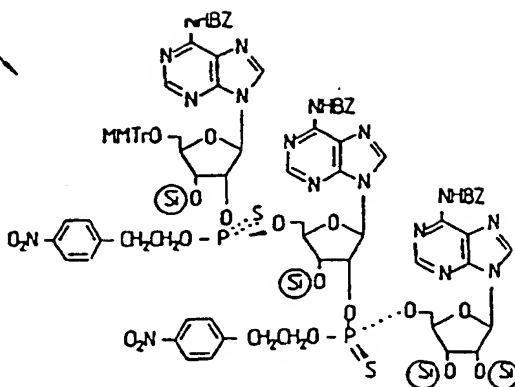
[0023] The trimer cores are prepared according to the following reaction scheme wherein "BZ" denotes the benzoyl radical "Si" denotes the *tert*-butyldimethylsilyl radical and "MMTr" represents the monomethoxytrityl radical. While not part of the invention, the preparation of the dimer core enantiomers *A_{Rp}A* (**6A**) and *A_{Sp}A* (**6B**) is included for completeness.



5 A

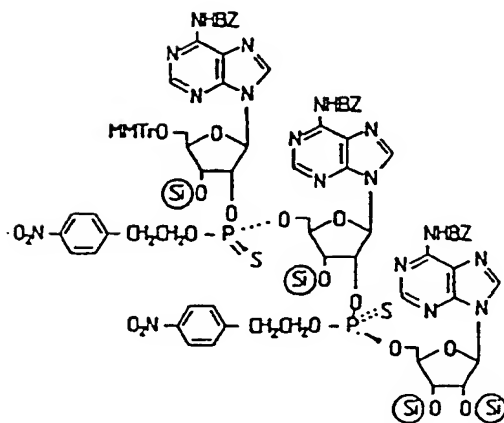


7A

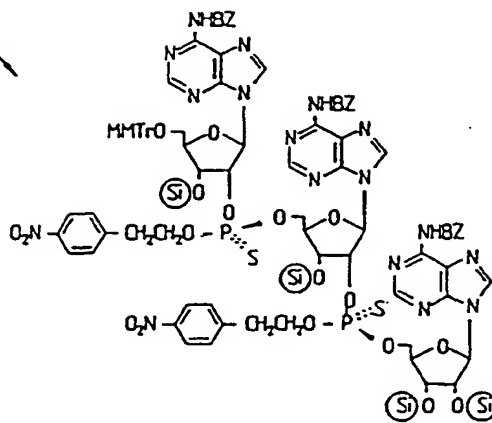


7B

5 B

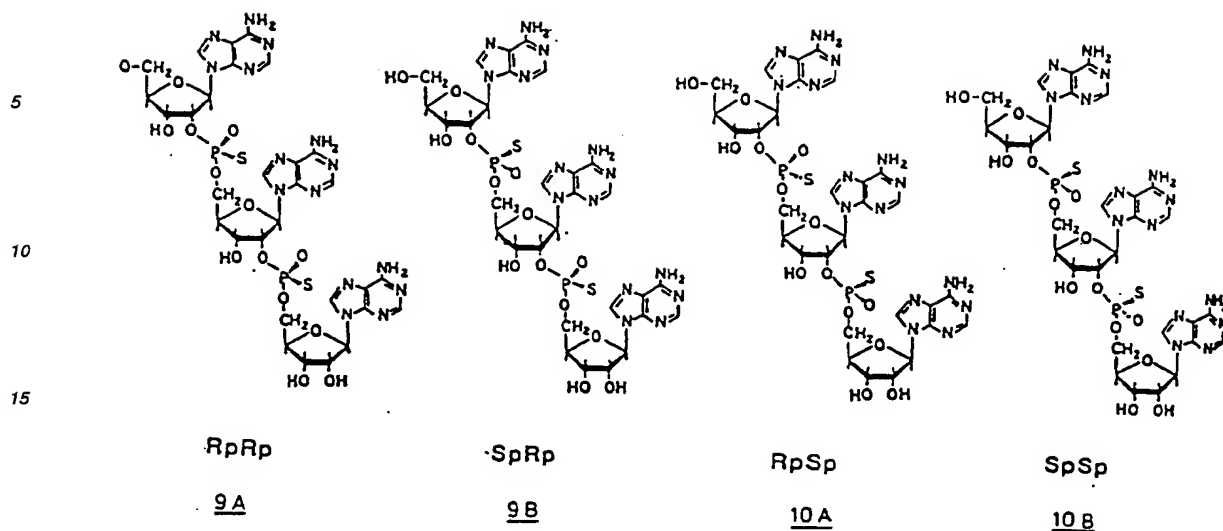


8A



8B

[0024] The blocking groups are removed from the fully-protected intermediates 7A, 7B, 8A, and 8B to yield the corresponding fully resolved trimer cores $A_{Rp}A_{Rp}A$ (9A), $A_{Sp}A_{Rp}A$ (9B), $A_{Rp}A_{Sp}A$ (10A) and $A_{Sp}A_{Sp}A$ (10B):



[0025] The compounds of the invention are advantageously prepared as soluble salts of sodium, ammonium or potassium. The preparative scheme begins with 6-N-benzoyl-3'-O-*tert*-butyldimethylsilyl-5'-O-monomethoxy-trityl-adenosine (compound 1E), which is advantageously prepared from adenosine according to the procedure of Flockerzi et al, *Liebigs Ann. Chem.*, 1568-1585 (1981).

[0026] Preparation of the compounds of the present invention is illustrated in more detail by reference to the following non-limiting examples. 3-Nitro-1,2,4-triazole; chlorooctahydroazonino-p-nitrophenylethoxyphosphate; 2,5-dichlorophenylphosphoro-dichloridate; and p-nitrophenylethanol used in the examples may be prepared advantageously from published procedures: Chattopadhyaya et. al. *Nucleic Acids Res.* 8:2039-2053 (1980); Schwarz et al., *Tetrahedron Lett.* 5513-5516 (1984); Uhlmann et al., *Helv. Chim. Acta* 64:1688-1703 (1981). These compounds are also available commercially in the United States. 2,5-Dichlorophenyl-phosphorodichloridate may be obtained from Fluka Chemical Corp., 980 S. Second St., Ronkonkoma, New York 11779, ("Catalog 15: Chemika-Biochemika" 1986/1987, no. 36212). 3-Nitro-1,2,4-triazole is available from Aldrich Chemical Co., P.O. Box 355, Milwaukee, WI 53201 (1986-1987 cat. no. 24,179.2). P-Nitrophenylethanol is available from Fluka Chemical Corp. (cat. no. 73,610). Chloro-octahydroazonino-p-nitrophenylethoxyphosphate may be prepared according to Example 1A, below.

[0027] Pyridine and triethylamine used in the examples were purified by distillation over KOH, tosyl chloride and calcium hydride. Dichloromethane was distilled over calcium chloride and then passed through basic alumina. Pure acetonitrile was obtained by distillation over calcium hydride.

[0028] Purification of the protected nucleotides was achieved by preparative column chromatography on silica gel 60 (0.063-0.2 mesh, Merck) and by preparative thick layer chromatography on silica gel 60 PF₂₅₄ (Merck). Thin layer chromatography ("TLC") was carried out on precoated thin layer sheets F 1500 LS 254 and cellulose thin layer sheets F 1440 from Schleicher & Scheull.

[0029] The starting material, 6-N-Benzoyl-3-O-*tert*-butyldimethylsilyl-5'-O-(4-monomethoxytrityl)adenosine (Compound 1E) and the reagent 6-N-benzoyl-2',3'-bis-O-(*tert*-butyldimethylsilyl)-adenosine (Compound 1G), are prepared according to Example 1.

EXAMPLE 1

[0030]

a. N⁶,N⁶,2',3',5'-O-Pentabenzoyl-adenosine: (1A) To a suspension of 5.34 g (20 mmole) adenosine (Sigma, dried at 80°C/10⁻³ Torr for 24 h) in 100 ml dry pyridine, 33.74 g (240 mmole) benzoyl chloride was added dropwise. After 20 h stirring at room temperature ("r.t.") the mixture was treated with 16 ml dry MeOH and then extracted with CHCl₃ (3 x 250 ml). The organic phase was washed with water (3 x 250 ml), dried over Na₂SO₄, and evaporated to dryness. Final coevaporation was performed with toluene. The residue was dissolved in CHCl₃/MeOH 2/1 by heating, and after cooling to r.t., petroleum ether (diethylether) was added until the solution became turbid. After standing for 12 h at 0°C 12.18 g was obtained, and from the mother liquor, 2.66 g of the product were isolated as colorless needles

of m.p. 183-184°C., yield 14.84 g (94%).

b. 6-N-Benzoyl adenosine (1B): A solution of 7.88 g (10 mmole) of the pentabenzoyl adenosine (1A) in 150 ml dry pyridine and 50 ml dry MeOH was treated with 50 ml 1 M sodium methylate solution. After 15 min the solution was poured onto an ice cold solution of a 110 ml DOWEX ion exchanger 50 x 4 (pyridinium form) in ca 20 ml water. After 5 h stirring the pH was 5.5-6.0. After filtering from the ion exchanger, the residue was washed with boiling MeOH/water (3/1). The filtrate was evaporated to dryness and crystallized from MeOH/water 2/1 to give 3.25 g (83%) of the product as colorless needles, m.p. 151-153°C.

c. 6-N-Benzoyl-5'-O-(4-methoxytrityl) adenosine (1C): 17.9 g (46 mmole) 6-N-benzoyl adenosine • H₂O (1B) was evaporated with dry pyridine (3 x 100 ml) and finally dissolved in 150 ml dry pyridine and 21.31 g (69 mmole) p-monomethoxytrityl chloride. The reaction solution was stirred at 50°C for 14 h and dry MeOH (50 ml) was added and allowed to come to r.t. The product was extracted with CHCl₃ (3 x 400 ml) and washed with water (3 x 400 ml). The organic phase was dried over Na₂SO₄ and evaporated to dryness. Final coevaporation was performed with toluene. Purification was performed using a silica gel column (16 x 2.5 cm, Merck) and eluted with 3 liter EtOAc/MeOH 7/3 to give 25.6 g (87%) of an amorphous powder. Crystallization was accomplished with acetone/water to yield the product, m.p. 120-125°C.

d. 6-N-Benzoyl-2'-O-(tert-butyldimethylsilyl)-5'-O-(4-methoxytrityl) adenosine (1D);

6-N-Benzoyl-3'-O-(tert-butyldimethylsilyl)-5'-O-(4-methoxytrityl) adenosine (1E);

6-N-benzoyl-2',3'-O-bis(tert-butyldimethylsilyl)-5'-O-(4-methoxytrityl) adenosine (1F):

To a solution of 4.05 g (26.9 mmole) tert-butyldimethylsilylchloride ("TBDMS-Cl") and 3.66 g (53.8 mmole) imidazole in 100 ml dry pyridine, were added 14.42 g (22.4 mmole) of compound 1C which was previously coevaporated with dry pyridine. After 15 h stirring at r.t., 5 ml dry MeOH was added and the mixture was evaporated to 1/3 volume. The crude product was extracted with CHCl₃ (3 x 250 ml) and washed with water. Upon evaporation, the crude product was first purified by a silicagel column (15 x 3 cm) with CH₂Cl₂/EtOAc (9/1), and subsequently purified using medium pressure chromatography (silicagel column, GSF-type C (N= 9000, V_D = 28 ml) at 8-10 bar pressure with the following mixtures of CH₂Cl₂/petrolether/EtOAc/EtOH: 100:100:10:0.5 (2 liters), first; 100/100/10/1 (3 liters), second; and 100/100/31/2 (0.5 liters), third. The title compounds were isolated. The retention time of the peak maxima for each compound was as follows: 25 min. for compound 1F (yield 1.84 g, 9%); 60 min. for compound 1D (yield 6.62 g, 39%); 105 min. for compound 1E (yield 8.32 g, 49%).

e. 6-N-Benzoyl-2',3'-bis-O-(tert-butyldimethylsilyl) adenosine (1G): 1.74 g (2 mmole) of compound 1D was stirred with 20 ml 80% acetic acid at 22°C. After 20 h the cleavage of the monomethoxytrityl group was complete. The reaction mixture was extracted with CHCl₃ (3 x 200 ml) and washed with 200 ml 1 M phosphate buffer (pH 7). The organic phase was dried over Na₂SO₄ and evaporated to dryness. Purification was accomplished using a silica gel column (2 x 10 cm) and eluted with CH₂Cl₂/MeOH (96/4). The light yellow product was dissolved in 5 ml CHCl₃ and treated with Et₂O until turbid. 0.982 g of the pure product crystallized out. The pure product crystallized out again from the mother liquor, 0.11 g, m.p. 189°C. The total yield was 1.092 g (91%).

EXAMPLE 1A

Chloro-octahydroazonino-p-nitrophenylethoxyphosphate

[0031]

a. P-nitrophenylphosphoric acid dichloride. To phosphorus trichloride (Fluka, N.Y., #79690) (28 ml, 0.317 moles) 80 ml anhydrous ether are added. The mixture is cooled to -30°C. P-nitrophenylethanol (8.35 g, 50 mmoles) is added, followed by stirring for 1.5 hr. Ether and excess PCl₃ is removed under vacuum to yield p-nitrophenylphosphoric acid dichloride (yield 80%).

b. Octahydroazonin. Caprylactam (Fluka, N.Y., #21631) (25 g, 117 mmoles) is combined with lithium aluminum hydride (10.5 g) in ether and reduced with stirring for 5 hr. The reaction mixture is filtered and evaporated with ether. The product is octahydroazonin (90% yield).

c. 1-Trimethylsilyl octahydroazonin. The silylamine of octahydroazonin is prepared by combining octahydroazonin (12.7 g, 0.1 moles) and trimethylsilane (0.12 moles) and 0.5 moles hexamethyldisilazane + 150 mg of ammonium sulfate. The mixture is refluxed for 90 hr and distilled under vacuum to yield 16.5 g of 1-trimethylsilyl octahydroazonin (82%).

d. Chloro-octahydroazonino-p-nitrophenylethoxyphosphate. P-nitrophenylphosphoric acid dichloride (26.8 g, 100 mmoles) and 1-trimethylsilyl octahydroazonin (19.9 g, 100 mmoles) are combined under nitrogen at 0°C. The mixture is warmed to room temperature and stirred 2-3 h. Trimethylsilyl dichloride is removed under vacuum. The product in the residue is chloro-octahydroazonino-p-nitrophenylethoxyphosphate (33.9 g, 94% yield).

EXAMPLE 2

[0032] 6-N-Benzoyl-3'-O-tert-butyldimethylsilyl-5'-O-(4-ethoxytrityl)-adenosine-2'-O-(p-nitrophenylethyl)-octahydroa-
 zonino-phosphoramidite (2). Compound 1E (0.758 g, 1.0 mmole) and diisopropylethylamine (0.52 g, 4 mmole) were
 dissolved in dichloromethane (5 ml) and chlorooctahydroazonino-p-nitrophenylethoxyphosphane (0.80 g, 2.22 mmole)
 was added dropwise. After stirring for 2 h at r.t., TLC analysis indicated complete reaction. The reaction mixture was
 transferred to a separatory funnel using saturated aqueous NaHCO₃ (50 ml) and the product was isolated by extraction
 with ethylacetate (2 x 50 ml). The organic layer was washed with saturated NaCl, dried (Na₂SO₄), and evaporated to
 dryness. The residue was dissolved in ethylacetate-triethylamine (95:5 v/v), chromatographed on a silica gel column
 (10 x 2 cm) previously calibrated with ethylacetate-triethylamine (9/1) and eluted with ethyl-acetate-triethylamine (95:5
 v/v). The product fractions were collected, evaporated to dryness, finally coevaporated with dichloromethane and dried
 in vacuo at 40°C to give compound 2 (1.05 g, 97%) [Anal. calcd. for C₅₉H₇₀N₇O₉PSi • 1 H₂O: C, 64.52; H, 6.60; N,
 8.92. Found: C, 63.93; H, 6.85; N, 8.62].

EXAMPLE 3

[0033] 6-N-Benzoyl-3'-O-tert-butyldimethylsilyl-5'-O-monoethoxytrityl-P-thioadenylyl-2'-[O^P-(p-nitrophenylethyl)-5']-6-
 N-benzoyl-2',3'-di-O-tert-butyldimethylsilyl-adenosine (4A + 4B). The phosphoramidite 2 (1.12 g, 1.0 mmole) and 6-N-
 benzoyl-2',3'-di-O-tert-butyldimethylsilyl-adenosine (3) (0.478 g, 0.7 mmole) were dried overnight in a drying pistol at 40°C
 in vacuo. The dried residue was then dissolved in dry acetonitrile (6 ml), and 3-nitro-1,2,4-triazole (0.285 g, 2.5 mmole)
 was added and stirred at r.t. for 3 h. Pyridine (6 ml) and sulfur (0.5 g) were added and after stirring at r.t. for 20 h, the
 reaction mixture was extracted with chloroform (300 ml). The organic phase was washed with saturated NaCl-solution
 (2 x 200 ml), dried (Na₂SO₄), and evaporated to dryness. Final evaporation was performed with toluene to remove pyri-
 dine. The residue was dissolved in chloroform and chromatographed on a silica gel column (15 x 2.5 cm) with 1 liter of
 chloroform to give a product fraction containing both Rp and Sp isomers. The separation of the diastereoisomers was
 carried out on preparative silica gel plates, using dichloromethane/ethylacetate/n-hexane (1:1:1 v/v). The plates were
 developed thrice. The higher Rf isomer (0.47 g; 42%, TLC in dichloromethane/ethylacetate/n-hexane, 1:1:1, 0.54) and
 the lower Rf isomer (0.31 g; 28%, TLC in dichloromethane/ethylacetate/n-hexane, 0.46) were obtained as colorless
 amorphous powders after drying at 40°C in vacuo. The higher Rf isomer was compound 4A. [Anal. calcd. for
 C₈₀H₉₈N₁₀O₁₄PSi₃ • 1 H₂O: C, 59.89; H, 6.32; N, 9.61. Found: C, 59.89; H, 6.32; N, 9.21]. ³¹P-NMR (400 MHz, CDCl₃,
 85% H₃PO₄, 69.841 ppm). The lower Rf isomer was compound 4B. [Anal. calcd. for C₈₀H₉₈N₁₀O₁₄PSi₃S • 1 H₂O: C,
 59.89; H, 6.28; N, 9.61. Found: C, 59.91; H, 4.61; N, 9.281. ³¹P-NMR (400 MHz, CDCl₃, 85% H₃PO₄, 69.223 ppm).

EXAMPLE 4

[0034] 6-N-Benzoyl-3'-O-tert-butyldimethylsilyl-P-thioadenylyl-2'-[O^P-(p-nitrophenylethyl)-5']-6-N-benzoyl-2',3'-di-O-
 tert-butyldimethylsilyl-adenosine (5A + 5B). 0.258 g, (0.164 mmole) of the pure isomers 4A and 4B, were detritylated
 separately by treatment of each with 2% p-toluenesulfonic acid in dichloromethane/methanol (4/1) (3.2 ml) at r.t. for 40
 min. The reaction mixture was diluted with chloroform (50 ml), washed with phosphate buffer, pH 7 (2 x 20 ml), and
 evaporated to a foam. The residue was purified by silica gel column chromatography (10 x 2.5 cm). Compounds 5A and
 5B were separated on the column by using chloroform and chloroform/methanol (100:0.5 till 100:1). The product frac-
 tions were collected and, after evaporation, dried in vacuo at 40°C to give in the case of 5A, 0.198 g (92%), and in the
 case of 5B, 0.192 g (89%). 5A has an Rf of 0.27 in dichloromethane/ethylacetate/n-hexane (1:1:1). [Anal. calcd. for
 C₆₀H₈₂N₁₁O₁₃PSi₃S • 1 H₂O: C, 54.15; H, 6.36; N, 11.57. Found: C, 53.78; H, 6.44; N, 11.72]. 5B has an Rf of 0.30 in
 the same system. [Anal. calcd. for C₆₀H₈₂N₁₁O₁₃PSi₃S: C, 54.90; H, 6.29; N, 11.73. Found: C, 54.90; H, 6.20; N,
 11.45].

EXAMPLE 5

[0035] P-Thioadenylyl-(2'-5')-adenosine (6A + 6B). The fully protected dimers 5A and 5B, respectively, were depro-
 tected separately by the following procedure. Each protected dimer (39 mg, 0.03 mmole) was treated with 0.5 M 1,8-
 diazabicyclo[5.4.0]undec-7-ene(1,5,5)(Fluka, cat. no. 33842, "DBU") in pyridine (9 ml), and after stirring at r.t. for 2 h
 was neutralized with 1 M acetic acid (4.5 ml) and finally evaporated. The residue was taken up in 1 M tetrabutylammo-
 nium fluoride ("Bu₄NF") in tetrahydrofuran ("THF") and after 24 h again evaporated to dryness. Deacetylation was
 achieved by treatment with conc. ammonia (20 ml) for 48 h followed by evaporation of the mixture. The residue was then
 dissolved in water (50 ml) and washed with chloroform (2 x 20 ml). The water phase was applied to a DEAE Sephadex
 A-25 column (60 x 1 cm) for elution with a linear gradient of a buffer of 0.001-0.25 M Et₃NH⁺HCO₃⁻, pH 7.5. The product
 was eluted at a concentration of 0.08-0.1 M. Evaporation to dryness followed by coevaporation with water (10 x 10 ml)

and final purification by paper chromatography with i-PrOH/conc. ammonia/water (7:1:2 v/v) yielded in the case of 6A 630 O.D. units (87.5%) and in the case of 6B 648 O.D. units (90%). The R_f of 6A on cellulose sheets, using the above system, was 0.29. The R_f of 6B was 0.30.

5 EXAMPLE 6

[0036] 6-N-Benzoyl-3'-O-tert-butylidimethylsilyl-5'-O-monomethoxytrityl-P-thioadenylyl-2'-[O^P-(p-nitrophenylethyl)-5'-N-6-benzoyl-3'-O-tert-butylidimethylsilyl-P-thioadenylyl-2'-[O^P-(p-nitrophenylethyl)-5'-6-N-benzoyl-2',3'-bis-O-tert-butylidimethylsilyl]adenosine (7A, 7B and 8A, 8B). The phosphitamide 2 (0.449 g; 0.41 mmole) was condensed with the 5'-hydroxy dimer 5A and 5B (0.0262 g; 0.2 mmole) separately in the presence of 3-nitro-1,2,4-triazole (0.114 g; 1.0 mmole) in dry acetonitrile (3.2 ml). After stirring at r.t. for 3 h, sulfur (0.2 g; 6.25 mmole) in pyridine (0.4 ml) was added for oxidation. After stirring at r.t. for another 24 h, the product was extracted with dichloromethane (50 ml), the organic phase was washed with saturated NaCl solution (2 x 20 ml), dried (Na₂SO₄), and then evaporated to dryness. Final coevaporation was performed with toluene to remove pyridine. The crude product was chromatographed on a silica gel column (15 x 2 cm) and eluted with chloroform/methanol (100:2) to give, on condensing with compound 5A, the isomer mixture 7A + 7B. The isomer mixture 8A + 8B, was obtained upon condensing the phosphitamide 2 with compound 5B in the same manner. The diastereomeric separation of each isomer mixture was accomplished by using preparative silica gel plates (20x20x0.2 cm) to which about 50 mg of isomer mixture per plate was applied for optimal separation. The plates were developed in dichloromethane/ethylacetate/n-hexane (1:1:0.5 v/v) three times. The appropriate bands were cut out and eluted with chloroform/methanol (4:1). The higher R_f isomer synthesized from 5A has a R_f of 0.58 in dichloromethane/ethylacetate/n-hexane (1:1:1) and yielded 48 % (0.218 g) of 7A. [Anal. calcd. for C₁₁₁H₁₃₅N₁₇O₂₂P₂Si₄S₂ · 1 H₂O: C, 57.56; H, 5.96; N, 10.28. Found: C, 57.38; H, 5.99; N, 10.11]. The lower R_f isomer has a R_f of 0.48 in the above-mentioned system, and yielded 34% (0.157 g) of 7B. [Anal. calcd. for C₁₁₁H₁₃₅N₁₇O₂₂P₂Si₄S₂ · 1 H₂O: C, 57.56; H, 5.96; N, 10.28. Found: C, 57.40; H, 5.97; N, 10.19]. The isomeric mixture derived from the 5'-hydroxy dimer 5B was separated in the same manner and yielded the higher R_f isomer 8A in 41% (0.186 g) with an R_f of 0.53 in the above solvent system. [Anal. calcd. for C₁₁₁H₁₃₅N₁₇O₂₂P₂Si₄S₂: C, 58.02; H, 5.92; N, 10.36. Found: C, 58.00; H, 5.84; N, 10.66]. The lower R_f isomer 8B showed a R_f value of 0.45 and yielded 35% (0.161 g). [Anal. calcd. for C₁₁₁H₁₃₅N₁₇O₂₂P₂Si₄S₂ · 1 H₂O: C, 57.56; H, 5.96; N, 10.28. Found: C, 57.30; H, 5.78; N, 10.03].

30 EXAMPLE 7

[0037] (Rp)-P-Thioadenylyl-(2'-5')-(Rp)-P-thioadenylyl-(2'-5')-adenosine (9A). A solution of 0.116 g (0.05 mmole) of the fully protected trimer 7A was detritylated with 2% p-toluenesulfonic acid in 1.5 ml dichloromethane/methanol (4:1) for 90 min. The mixture was dissolved in CHCl₃, washed with phosphate buffer (2x15 ml), dried (Na₂SO₄), and evaporated to dryness. The residue was chromatographed on silica gel plates (20x20x0.2 cm), developed with dichloromethane/ethylacetate/n-hexane (5:5:3 v/v). The product band (R_f 0.35) was cut out, eluted with chloroform/methanol (7:5) and gave on evaporation to a colorless foam a yield of 70-83%. 38.5 mg (18.9 micromole) of the product was then stirred with 0.5 M DBU in pyridine (7.5 ml) for 20 h, neutralized with 1M acetic acid (3.75 ml) and finally evaporated. The evaporated product was desilylated through treatment with 1M Bu₄NF in THF (6 ml) for 24 h. The mixture was concentrated in vacuo. The residue was dissolved in conc. ammonia (25 ml) and stirred at r.t. for 48 h. After evaporating the solution, the residue was taken up in water (20 ml) and washed with chloroform (2x10 ml). The aqueous phase was put on a DEAE Sephadex A-25 column (60x1 cm) and the product was eluted with a linear gradient of Et₃NH⁺HCO₃⁻-buffer. The product fraction was collected, evaporated and further purified by paper chromatography using i-PrOH/conc. ammonia/water (6:1:3) to give the title compound in 75-80% yield.

45 EXAMPLE 8

[0038] (Sp)-P-Thioadenylyl-(2'-5')-(Rp)-P-thioadenylyl-(2'-5')-adenosine (9B). A solution of 0.116 g (0.05 mmole) of the fully protected trimer 7B was subjected to the procedure of Example 7. The title compound was obtained in 75-80% yield.

EXAMPLE 9

[0039] (Rp)-P-Thioadenylyl-(2'-5')-(Sp)-P-thioadenylyl-(2'-5')-adenosine (10A). A solution of 0.116 g (0.05 mmole) of the fully protected trimer 8A was subjected to the procedure of Example 7. The title compound was obtained in 75-80% yield.

EXAMPLE 10

[0040] (Sp)-P-Thioadenylyl-(2'-5')-(Sp)-P-thioadenylyl-(2'-5')-adenosine (10B). A solution of 0.116 g (0.05 mmole) of the fully protected trimer 8B was subjected to the procedure of Example 7. The title compound was obtained in 75-80% yield.

[0041] The UV-absorption spectra in methyl alcohol and ¹H-NMR spectra of the above-prepared protected monomer, dimer and trimer cores are set forth in Tables 1 and 2, respectively.

TABLE 1

UV-Absorption Spectra of Protected Monomer, Dimer and Trimer Cores in MeOH				
Compound	λ_{\max} (nm)		lgε	
<u>2</u>	230	277	4.47	4.50
<u>4A</u>	231	277	4.66	4.70
<u>4B</u>	231	277	4.66	4.70
<u>5A</u>		278		4.70
<u>5B</u>		278		4.70
<u>7A</u>	230	278	4.78	4.90
<u>7B</u>	230	278	4.78	4.89
<u>8A</u>	230	278	4.79	4.90
<u>8B</u>	230	278	4.78	4.90

TABLE 2

¹ H-NMR Spectra of Protected Monomer, Dimer and Trimer Cores ¹				
Compound	1'-H	2-H	8-H	Solvent
<u>2</u>	6.11 6.13	8.70	8.23	CDCl ₃
<u>4A</u>	6.30d 5.87d	8.68; 8.60	8.19; 8.17	CDCl ₃
<u>4B</u>	6.29d 5.94d	8.72; 8.62	8.26; 8.18	CDCl ₃
<u>5A</u>	6.06d 5.94d	8.82; 8.75	8.25; 8.08	CDCl ₃
<u>5B</u>	6.13d 5.90d	8.74; 8.73	8.26; 8.24	CDCl ₃
<u>7A</u>	6.23d 6.08d 5.84d	8.69; 8.58; 8.55;	8.20; 8.11; 8.01	CDCl ₃
<u>7B</u>	6.22d 6.17d 5.85d	8.67; 8.60; 8.57;	8.23; 8.10; 8.00	CDCl ₃
<u>8A</u>	6.27d 6.13d 5.93d	8.71; 8.61; 8.60;	8.21; 8.13; 8.00	CDCl ₃
<u>8B</u>	6.27d 6.22d 5.90d	8.71; 8.64; 8.61;	8.27; 8.22; 8.19	CDCl ₃

¹ δ values in ppm; Standard TMS; characteristic signals

Assignment of Absolute Configuration of 2',5'-Phosphorothioate Adenylate Trimer Cores

[0042] Determination of the absolute configurations of the trimer cores was accomplished by ³¹P-NMR fast bombardment mass spectrometry and enzymatic digestion.

[0043] It is known that the enzyme SVPD preferentially cleaves Rp-3',5'- or 2',5'-phosphorothioate linkages from the 2'/3'-terminus. Nelson et al, J. Org. Chem. 49:2314-2317 (1984); Eppstein et al, J. Biol. Chem. 261:5999-6003 (1986); Lee et al, Biochemistry 24:551-555 (1985). SVPD hydrolysis of the chemically synthesized dimer core A_{RP}A yielded

adenosine and AMPS in a molar ratio of 1:1, respectively; the half-life was 3 hours (Table 3). The A_{Sp}A dimer core was not a substrate for SVPD under these conditions. Trimer core 9A has the RpRp internucleotide linkage configuration as determined by hydrolysis by SVPD to yield AMPS Plus A_{Rp}A in a molar ratio of 1:1, respectively. Similarly, SVPD hydrolysis of trimer core 9B yielded A_{Sp}A and AMPS, thus identifying trimer core 9B as having the SpRp internucleotide linkage configuration (Table 3). Trimer cores 10A and 10B were not substrates for SVPD (Table 3), revealing the presence of the Sp configuration in the internucleotide linkage adjacent to the 2'/3'-termini.

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TABLE 3 Analytical Data, Dimer and Trimer 2,5-Phosphorothioate Adenylate Cores

2,5-Phosphorothioate		Hydrolysis by:					
31P-NMR (PPM ^a)	R _T (min ^b)	SVPD		L cell extract		serum phospho-	Stereo- config- uration assigned
		dimer core isolated	half life	dimer core; half life isolated	dimer core half life isolated	diesterases	
Dimer Cores							
<u>6A</u>	57.63	19.5	3 h	not cleaved	not cleaved	<u>RP</u>	
<u>6B</u>	56.13	24.2	not cleaved	not cleaved	not cleaved	<u>SP</u>	
Trimer Cores							
<u>9A</u>	57.45, 57.71	30.5	<u>RP</u>	1 h	<u>RP</u>	18 h <u>RP</u>	<u>RP</u> <u>RP</u> ^b
<u>9B</u>	57.55, 56.62	33.0	<u>SP</u>	8 h	<u>SP</u>	15 h not cleaved	<u>SP</u> <u>RP</u> ^c
<u>10A</u>	56.34, 57.54	35.2	not cleaved	not cleaved	<u>RP</u>	20 days not cleaved	<u>RP</u> <u>SP</u> ^c
<u>10B</u>	56.50, 56.26	39.5	not cleaved	not cleaved	not cleaved	not cleaved	<u>SP</u> <u>SP</u> ^b
A ₃ core	14.5	--	5 min	--	10 min	10 min	
A ₂ core			ND	--	10 min	ND	

^adecoupled spectra^bassignment confirmed by coupled and decoupled ³¹P-NMR^cassignment confirmed by coupled ³¹P-NMR and enzymatic hydrolyses^dHPLC retention times

[0044] The four 2,5-phosphorothioate adenylate trimer cores were further characterized by hydrolysis with the enzyme 2'-phosphodiesterase ("2'-PDE"), an exoribonuclease found in L cell extract. The enzyme cleaves from the

2'/3'-terminus. Whereas authentic A_2 and A_3 cores were hydrolyzed to adenosine and AMP with a half-life of 10 min, the dimer cores $A_{Rp}A$ and $A_{Sp}A$ were not substrates for 2'-PDE (Table 3). The fact that the 2,5-phosphorothioate dimer cores were not substrates for 2'-PDE (unlike authentic 2-5A) greatly assisted in the assignment of the stereoconfigurations of the 2',5'-phosphorothioate adenylate trimer cores. Trimer core 9A was a substrate for 2'-PDE; the products of hydrolysis were $A_{Rp}A$ and AMPS. Trimer core 9B was a substrate for SVPD, yielding $A_{Sp}A$ and AMPS; trimer core 10A was a substrate, yielding $A_{Rp}A$ and AMPS; trimer 10B was not a substrate for 2'-PDE.

[0045] ^{31}P -NMR spectroscopy has revealed that Sp stereoisomers of phosphorothioates resonate to higher field than Rp diastereomers. Further, Sp diastereomers have a longer retention time on reverse phase HPLC than Rp diastereomers. The $A_{Sp}A$ dimer core resonates upfield from the $A_{Rp}A$ (Table 3). Similarly, two singlets observed for $A_{Sp}A_{Sp}A$ resonate upfield from the two singlets observed for $A_{Rp}A_{Rp}A$ (Table 3). Assignment of the absolute configurations of trimer cores 9A ($RpRp$) and 10B ($SpSp$) was based on the two singlets which resonate at the same frequency as the singlets observed for the $A_{Rp}A$ and $A_{Sp}A$ dimer cores. Assignment of configurations for trimer cores 9B and 10A was made in combination with the enzyme degradations and HPLC analyses (Table 3). The ^{31}P -NMR spectra revealed that the δ ppm between the two singlets for the $A_{Sp}A_{Rp}A$ trimer core is 1.2, whereas the two singlets for the $A_{Rp}A_{Sp}A$ have a δ ppm of 0.8 (assignment is 5' to 2'/3' terminus).

[0046] The metabolic stability of the 2,5'-phosphorothioate dimer and trimer cores is markedly greater than authentic 2-5A. The rate of hydrolysis of the trimer cores by the 3'-exonuclease SVPD is, in order of decreasing stability: $A_{Sp}A_{Rp}A > A_{Rp}A_{Rp}A > A_3$. The trimer cores $A_{Rp}A_{Sp}A$ and $A_{Sp}A_{Sp}A$ are not substrates of SVPD (Table 3). The 3'→5' direction of stepwise cleavage by SVPD is blocked by an Sp configuration, preventing cleavage of the upstream adjacent phosphorothioate linkage. With 2'-PDE, the $A_{Rp}A_{Rp}A$, $A_{Sp}A_{Rp}A$ and $A_{Rp}A_{Sp}A$ trimer cores, but not the $A_{Sp}A_{Sp}A$ trimer core, were substrates. With both SVPD and 2'-PDE, the dimer cores (either $A_{Rp}A$ or $A_{Sp}A$) accumulate following hydrolysis of the $A_{Rp}A_{Rp}A$, $A_{Sp}A_{Rp}A$ and $A_{Rp}A_{Sp}A$ trimer cores. Hydrolysis of the 2-5A molecule by SVPD and 2'-PDE proceeds from the 2'/3'-terminus. Therefore, introduction of the phosphorothioate group into the trimer core results in the accumulation of the $A_{Rp}A$ or $A_{Sp}A$ dimer cores from the 5'-terminus and the accumulation of AMPS from the 2'/3'-terminus (Table 3). With authentic A_3 , there is no detectable accumulation of A_2 following hydrolysis by SVPD or 2'-PDE.

[0047] None of the Sp linkage-containing trimer cores were cleaved by SVPD. While the half-life of $A_{Sp}A_{Rp}A$ upon cleavage by L-cell extract (15 hours) did not differ significantly from that of $A_{Rp}A_{Rp}A$ (18 hours), the half-life for the $A_{Rp}A_{Sp}A$ trimer was orders of magnitude longer (20 days). $A_{Sp}A_{Sp}A$ was not cleaved by L-cell extract (Table 3).

Preparation of Phosphorothioate Tetramer Cores

[0048] The following non-limiting examples illustrate the preparation of the fully-resolved tetramer core compounds of the invention.

EXAMPLE 11

[0049]

- a. (Sp)-P-Thioadenylyl-(2'-5')-(Sp)-P-thioadenylyl-(2'-5')-(Sp)-P-thiodenylyl-(2'-5')-adenosine
- b. (Rp)-P-thiodenylyl-(2'-5')-(Sp)-P-thioadenylyl-(2'-5')-(Sp)-P-thiodenylyl-(2'-5')-adenosine.

[0050] A solution of 0.149 g (0.065 mmole) of fully protected trimer 8B, which has the stereoconfiguration $SpSp$, was detritylated with 2% p-toluenesulfonic acid in 1.5 ml dichloromethane/methanol (4:1) for 3 h at room temperature. The mixture was diluted with 50 ml $CHCl_3$, washed with phosphate buffer (2 x 15 ml), dried (Na_2SO_4), and evaporated to dryness. The residue was chromatographed on silica gel plates (20 x 20 x 0.2 cm) developed with dichloromethane/ethylacetate/n-hexane (5:5:3 v/v). The product band, R_f 0.55, was cut out, eluted with chloroform/ethanol (1:1) and gave, on evaporation to a colorless foam of 0.108 g (yield 88%). The 5'-deblocked $SpSp$ trimer 8B (101 mg; 0.05 mM) was dissolved in 0.5 ml acetonitrile overnight with phosphitamide 2 (0.105 g; 0.1 mmole). 3-Nitro-1,2,4-triazole (0.023 g; 0.2 mmole) was added. After stirring at room temperature for 3 h, sulfur (0.042 g; 1.3 mmole) in pyridine (0.084 ml) was added for oxidation. After stirring at room temperature for another 24 h, the product was extracted with dichloromethane (50 ml), the organic phase was washed with saturated NaCl solution (2 x 20 ml), dried over Na_2SO_4 , and then evaporated to dryness. Final coevaporation was performed with toluene to remove pyridine. The crude product was purified by using preparative silica gel plates (20 x 20 x 0.2 cm) to which about 50 mg per plate was applied for optimal separation by developing in dichloromethane/ethylacetate/n-hexane (1:1:0.5 v/v) three times. The band containing the fully protected $SpSpSp$ tetramer (R_f 0.3) and the band containing the fully protected $RpSpSp$ tetramer (R_f 0.4), were cut out and eluted with chloroform/methanol (4:1). The yield of the fully protected $SpSpSp$ tetramer was 43 mg; 29%. The yield of the fully protected $RpSpSp$ compound was 53 mg; 35.5%. The two isomers (7.3 micromoles;

0.22 mg) were deblocked by stirring with 0.5 M DBU in pyridine (5.0 ml) for 20 h, neutralized with 1M acetic acid/ pyridine (0.5 ml) and finally evaporated. The subsequent desilylation was achieved with 1M tetrabutylammonium fluoride in tetrahydrofuran (3.6 ml) for 48 h at r.t. The mixture was concentrated in vacuo, the residue dissolved in conc. ammonia (15 ml) and stirred at r.t. for 48 h. After evaporating the solution, the residue was taken up in 5 ml of 80% acetic acid and allowed to stand for 20 h at r.t. The residue was dissolved in about 5 ml water and put on a DEAE Sephadex A-25 column (60 x 1 cm) and the product was eluted with a linear gradient of $\text{Et}_3\text{NH}^+\text{HCO}_3^-$ buffer (pH 7.5) (gradient 0.001 - 1 M). The product fractions were collected, evaporated and further purified by paper chromatography using; i-PrOH/conc. ammonia/water (6:1:3). The tetramer isomers were eluted with water to give $\text{A}_{\text{Sp}}\text{A}_{\text{Sp}}\text{A}_{\text{Sp}}\text{A}$ (72% yield; R_f 0.23) and $\text{A}_{\text{Rp}}\text{A}_{\text{Sp}}\text{A}_{\text{Sp}}\text{A}$ (73% yield; R_f 0.29) as the ammonium salt.

EXAMPLE 12

[0051]

- a. (Rp)-P-Thioadenylyl-(2'-5')-(Rp)-P-thioadenylyl-(2'-5')-(Rp)-P-thioadenylyl-(2'-5')-adenosine
 b. (Sp)-P-Thioadenylyl-(2'-5')-(Rp)-P-thioadenylyl-(2'-5')-(Rp)-P-thioadenylyl-(2'-5')-adenosine

[0052] The title compounds are prepared by following the procedure of Example 11, but substituting the fully protected trimer 7A for 8B as the starting material.

EXAMPLE 13

[0053]

- a. (Rp)-P-Thioadenylyl-(2'-5')-(Sp)-P-thioadenylyl-(2'-5')-(Rp)-P-thioadenylyl-(2'-5')-adenosine
 b. (Sp)-P-Thioadenylyl-(2'-5')-(Sp)-P-thioadenylyl-(2'-5')-(Rp)-P-thioadenylyl-(2'-5')-adenosine

[0054] The title compounds are prepared by following the procedure of Example 11, but substituting the fully protected trimer 7B for 8B as the starting material.

EXAMPLE 14

[0055]

- a. (Sp)-P-Thioadenylyl-(2'-5')-(Sp)-P-thioadenylyl-(2'-5')-(Sp)-P-thioadenylyl-(2'-5')-adenosine
 b. (Sp)-P-Thioadenylyl-(2'-5')-(Sp)-P-thioadenylyl-(2'-5')-(Sp)-P-thioadenylyl-(2'-5')-adenosine

[0056] The title compounds are prepared by following the procedure of Example 11, but substituting the fully protected trimer 8A for 8B as the starting material.

Preparation of 2',5'-Phosphorothioate Oligoadenylylate 5'-Monophosphates

[0057] 5'-Monophosphates of 2',5'-oligoadenylylates are readily prepared by reacting the corresponding core compounds with POCl_3 . Such treatment would result in the elimination of sulfur from the phosphorothioate internucleotide linkages of the compounds of the present invention, and the formation of 2-5A. Thus, the 5'-monophosphates of the phosphorothioate oligoadenylylates must be prepared from the corresponding fully protected core compounds from which the monomethoxytrityl blocking group on the 5'-terminal nucleotide has been removed. The conditions of the phosphorylation must be such that the p-nitrophenylethyl blocking groups on the internucleotide phosphorous atoms remain intact.

[0058] The 5'-monophosphate of each resolved trimer core of the present invention was prepared from the 5'-hydroxy analogue of the corresponding fully protected trimer 7A, 7B, 8A or 8B. The intermediate 5'-phosphotriester (11A, 11B, 12A or 12B) was prepared according to Example 15 and then freed of all blocking groups according to Example 16 to yield the 5'-monophosphate. The procedure of Examples 15 and 16 may be used for forming the 5'-monophosphate of any of the four trimer core stereoisomers.

EXAMPLE 15

[0059] 5'-O-(2,5-Dichlorophenyl-p-nitrophenylethyl)-phosphoryl-6-N-benzoyl-3'-O-tert-butyldimethylsilyl-P-thioadeny-

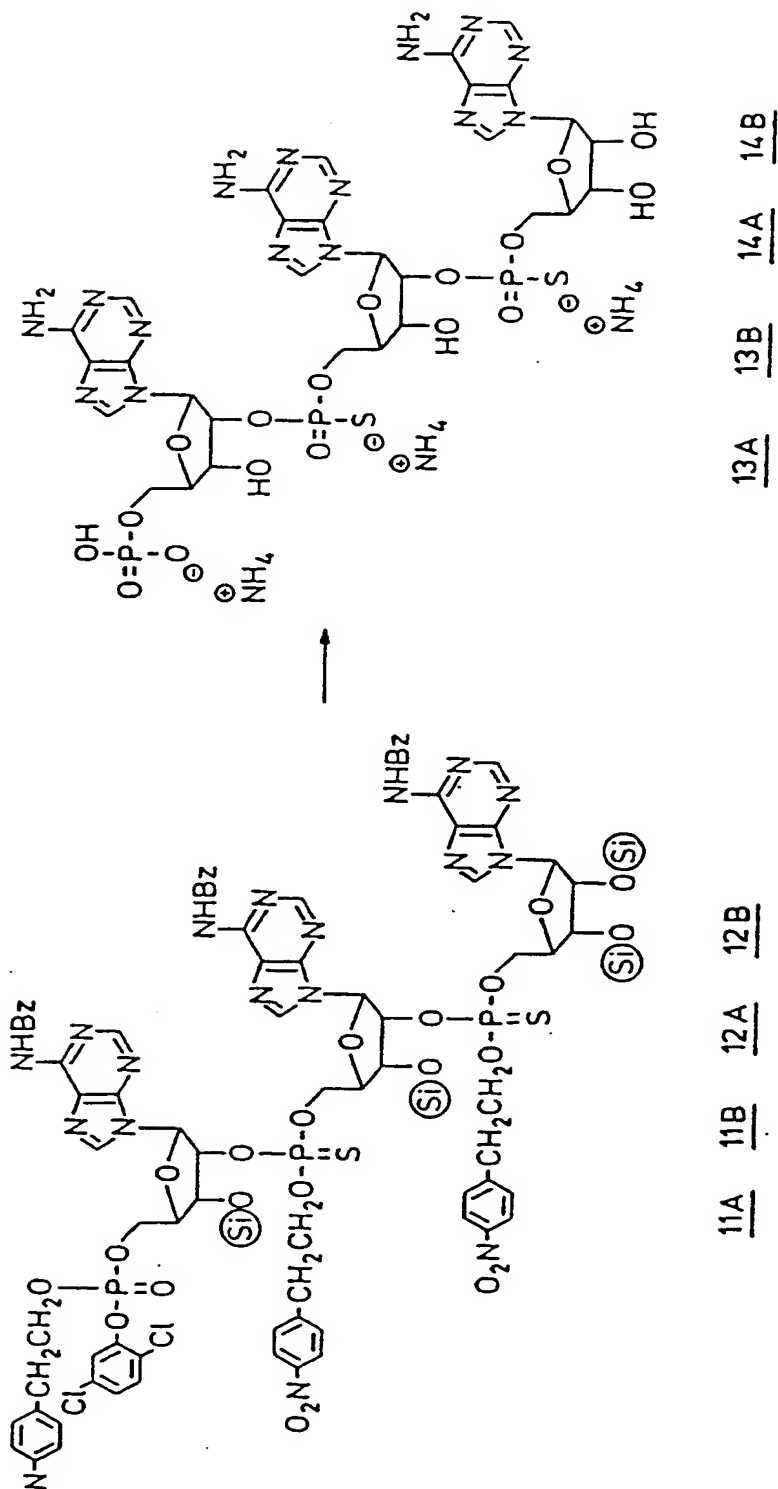
lyl-2'-[O^P-(p-nitrophenylethyl)-5']-6-N-benzoyl-3'-O-tert-butylidimethylsilyl-P-thioadenylyl-2'-[O^P-(p-nitrophenylethyl)-5']-6-N-benzoyl-2',3'-di-O-tert-butylidimethylsilyl adenosine (11A, 11B, 12A or 12B): To a solution of 1,2,4-triazole (0.011 g; 0.16 mmole) and 2,5-dichlorophenylphosphorodichloridate (0.022 g; 0.078 mmole) in dry pyridine (0.5 ml) was added the 5'-deblocked analogue of either 7A, 7B, 8A or 8B (0.1 g; 0.049 mmole) (prepared as an intermediate in Example 11), and after stirring for 30 min, p-nitrophenylethanol (0.02g; 0.119 mmole) was added and stirring continued for 20 h. The solution was then extracted with chloroform (50 ml), the organic phase was washed with water (2 x 20 ml), evaporated to dryness, and finally co-evaporated with toluene. The residue was purified by silica gel chromatography on preparative plates (20 x 20 x 0.2 cm) using the system dichloromethane/n-hexane/ethylacetate (1:1:1 v/v). The product band was eluted with chloroform/methanol (4:1) and evaporated in vacuo to give 11A, 11B, 12A or 12B in 70-80% yield, respectively.

EXAMPLE 16

[0060]

- a. 5'-O-Phosphoryl-(Rp)-thioadenylyl-(2'-5')-(Rp)-thioadenylyl-(2'-5')-adenosine (13A)
- b. 5'-O-Phosphoryl-(Sp)-thioadenylyl-(2'-5')-(Rp)-thioadenylyl-(2'-5')-adenosine (13B)
- c. 5'-O-Phosphoryl-(Rp)-thioadenylyl-(2'-5')-(Sp)-thioadenylyl-(2'-5')-adenosine (14A)
- d. 5'-O-Phosphoryl-(Sp)-thioadenylyl-(2'-5')-(Sp)-thioadenylyl-(2'-5')-adenosine (14B)

[0061] p-Nitrobenzaloxime (0.036 g; 0.216 mmole) was stirred for 30 min in dioxane/triethylamine/water (each 0.5 ml), the appropriate 5'-phosphotriester 11A, 11B, 12A or 12B (0.05 g, 0.02 mmole) was added and the mixture was kept at r.t. for 4 h. The solution was evaporated to dryness, followed by coevaporation with toluene (2x5 ml), and the residue purified by preparative TLC on plates (20x20x0.2 cm) in chloroform/methanol (95:5). The product band was eluted with chloroform/methanol/triethylamine (5:1:1) and evaporated to dryness. This material (0.022 g; 10 micromole) was stirred with 0.5 M DBU in pyridine (8 ml) at r.t. for 24 h, the solution neutralized with 1M acetic acid (4 ml) and evaporated to dryness. The residue was treated with 1M Bu₄NF in THF (6 ml) for 48 h and after evaporation the debenzoylation was accomplished by treatment with conc. ammonia (25 ml) at r.t. for 48 h. The solution was evaporated. The deblocked crude trimer 5'-monophosphate was taken up in water (25 ml) and washed with chloroform (2x10 ml). The aqueous phase put on a DEAE Sephadex A-25 column (60x1 cm) for elution with a linear gradient of 0.001-1M Et₃NH⁺HCO₃⁻ buffer. The product fractions were collected, evaporated to dryness, and after several coevaporations with water were further purified by paper chromatography using the i-PrOH/conc ammonia/water-system (55:10:35). The product band was eluted with water and gave on lyophilization the trimeric P-thioadenylate 5'-monophosphate 13A, 13B, 14A or 14B as ammonium salts in 68-74% yield.



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TABLE 4

¹ H-NMR Spectra of 5'-O-Phosphoryl-P-thioadenylyl-(2'-5')-P-thioadenylyl-(2'-5')-adenosine Stereoisomers ²				
Compound	1'-H	2-H	8-H	Solvent
13A	6.04 5.92d 5.75d	8.31; 8.18; 8.12;	7.98; 7.85; 7.80	D ₂ O
13B	6.12s 5.94d 5.76d	8.26; 8.21; 8.11;	7.99; 7.94; 7.86	D ₂ O
14A	6.03s 5.92d 5.80d	8.27; 8.22; 8.15;	8.04; 7.93; 7.81	D ₂ O
14B	6.09s 5.94s 5.81d	8.41; 8.26; 8.14;	8.07; 8.02; 7.89	D ₂ O

² δ values in ppm; Standard TMS; characteristic signals

[0063] Monophosphorylation of the 5'-deblocked protected trimers to form the 5'-phosphotriesters 11A, 11B, 12A or 12B proceeds in high yield, 70-80%, followed by the further high yield (68-74%) step of complete deprotection resulting in the trimer 5'-monophosphates 13A, 13B, 14A or 14B.

[0064] The 5-monophosphates of each resolved tetramer core compound of the present invention is prepared in the same fashion, using the identical molar quantities as in Examples 15 and 16 except that the starting material for the synthesis is the 5'-hydroxy analogue of the fully protected tetramer rather than the 5'-hydroxy analogue of the fully protected trimer. The following fully resolved tetramer 5'-monophosphates are thus prepared:

5'-O-Phosphoryl-(Rp)-thioadenylyl-(2'-5')-(Rp)-thioadenylyl-(2'-5')-(Rp)-thioadenylyl-(2'-5')-adenosine
 5'-O-Phosphoryl-(Rp)-thioadenylyl-(2'-5')-(Rp)-thioadenylyl-(2'-5')-(Sp)-thioadenylyl-(2'-5')-adenosine
 5'-O-Phosphoryl-(Rp)-thioadenylyl-(2'-5')-(Sp)-thioadenylyl-(2'-5')-(Sp)-thioadenylyl-(2'-5')-adenosine
 5'-O-Phosphoryl-(Sp)-thioadenylyl-(2'-5')-(Sp)-thioadenylyl-(2'-5')-(Sp)-thioadenylyl-(2'-5')-adenosine
 5'-O-Phosphoryl-(Sp)-thioadenylyl-(2'-5')-(Rp)-thioadenylyl-(2'-5')-(Sp)-thioadenylyl-(2'-5')-adenosine
 5'-O-Phosphoryl-(Sp)-thioadenylyl-(2'-5')-(Rp)-thioadenylyl-(2'-5')-(Rp)-thioadenylyl-(2'-5')-adenosine
 5'-O-Phosphoryl-(Sp)-thioadenylyl-(2'-5')-(Sp)-thioadenylyl-(2'-5')-(Rp)-thioadenylyl-(2'-5')-adenosine
 5'-O-Phosphoryl-(Rp)-thioadenylyl-(2'-5')-(Sp)-thioadenylyl-(2'-5')-(Rp)-thioadenylyl-(2'-5')-adenosine

Preparation of 2',5'-Phosphorothioate Oligoadenylylate 5'-Diphosphates and 5'-Triphosphates

[0065] The 5'-diphosphate and 5'-triphosphate of the 2',5'-phosphorothioate oligoadenylylates may be prepared from the 5'-monophosphate by following the procedure of Example 17.

EXAMPLE 17

[0066] All reactions are performed in glassware oven-dried at 125°C for 18-24 hr. A 2',5'-phosphorothioate oligoadenylylate stereoisomer (trimer or tetramer, 400 OD units at 260 nm) is dissolved in 500 microliters of dry dimethylformamide ("DMF") and dried in vacuo in a 10 ml conical flask at 35°C. This process is repeated three times. To the dry residue, 50 micromoles of triphenylphosphine, 100 micromoles of imidazole and 50 micromoles of dipyridinyl disulfide are added. The mixture is dissolved in 500 microliters dry DMF plus 50 microliters of dry dimethylsulfoxide. The solution is stirred with a stirring bar for 2 hr at room temperature. After 2 hr the solution is homogeneous (after 30 minutes, the solution begins to change to yellow). The solution is transferred dropwise to 10 ml of a 1% NaI/dry acetone (w/v) solution. The clear white precipitate which forms is the sodium salt of the 5'-phosphoroimidazolidate. The precipitate is centrifuged at room temperature, the supernatant is decanted, and the precipitate is washed three times with 10 ml dry acetone. The centrifuging is repeated. The precipitate is dried over P₂O₅ in vacuo for 2 hr. The precipitate is dissolved in 200 microliters of freshly prepared 0.5 M tributylammonium pyrophosphate in dry DMF. The solution is maintained at room temperature for 18 hr after which time the DMF is removed in vacuo. The residue is dissolved in 0.25 M triethylammonium bicarbonate buffer ("TEAB") (pH 7.5). The 5'-di and 5'-triphosphate products are separated using a DEAE-Sephadex A25 column (HCO₃⁻ form; 1 x 20 cm) with a linear gradient of 0.25 M to 0.75 M TEAB. Fractions (10 ml) are collected. The product is observed by ultraviolet spectroscopy at 254 nm. The fractions containing the 5'-di- and 5'-triphosphates are separately pooled and dried in vacuo. The TEAB is removed by repeated addition of water followed by lyophilization. The yield of the 5'-diphosphate is about 5%; the yield of the 5'-triphosphate is about 60%.

[0067] It is generally regarded that activation of RNase L by 2-5A is key to the antiviral defense mechanisms. Interferon induces transcription of the enzyme 2-5A synthetase which produces 2',5' linked oligoadenylylates upon activation

of double-stranded RNA. The only known biochemical effect of 2-5A is activation of RNase L. This enzyme hydrolyses mRNA and rRNA, thereby resulting in inhibition of protein synthesis. The activation of RNase L is transient unless 2-5A is continuously synthesized, since 2-5A is rapidly degraded. RNase L activation thus plays a critical role in inhibiting replication, and therefore in defending against infection by viruses.

5 [0068] According to the invention, all four of the 2',5'-phosphorothioate adenylylate trimer cores, and their 5'-monophosphates bind to RNase L, as determined by radio binding assay according to the method of Knight et al, *Meth. Enzymol.* 79: 216-227 (1981). The 2',5'-phosphorothioate adenylylate trimer cores and authentic A_3 were able to displace $p_3A_4[^{32}P]pCp$ probe from RNase L in L929 cell extracts in a concentration-dependent manner (Fig. 1A). IC_{50} s varied from 2×10^{-6} to 5×10^{-6} M. However, the 5'-monophosphorylated trimers had 1000-fold higher binding affinity to RNase L than their respective cores, that is, IC_{50} s ranged from 2×10^{-9} to 5×10^{-9} M (Fig. 1A). Without wishing to be bound by any theory, this increase may be attributed to the ability of the 5'-monophosphates to anchor the molecule to RNase L more effectively because of increased polarity.

[0069] The 2',5'-phosphorothioate cores, with one exception, have the correct stereoconfiguration to activate RNase L. The activation of partially-purified RNase L by the 2',5'-phosphorothioates was measured according to the core-cellulose assay of Silverman, *Analyt. Biochem.* 144:450-460 (1985) which relies on hydrolysis of the substrate poly(U)-3- $[^{32}P]pCp$. Surprisingly, three of the four 2',5'-phosphorothioate adenylylate cores were able to activate RNase L to cleave poly(U)-3- $[^{32}P]pCp$ in the core-cellulose assay. (Figure 1B) The order of activation of the trimer cores and the corresponding 5'-monophosphates is: $RpRp>SpRp>RpSp$ (Fig. 1B). While $pA_{Rp}A_{Rp}A$ was the most efficient activator of RNase L, the compound is metabolically unstable and is readily attacked by phosphodiesterases (See Table 3). The $SpSp$ trimer core did not activate RNase L, even at a concentration of 10^{-3} M. As was observed in the binding assay (Fig. 1A), there was a 1000-fold increase in the activation of RNase L by the 5'-monophosphates of the 2',5'-phosphorothioate trimers compared to their respective cores.

[0070] Activation of RNase L by 2',5'-phosphorothioate adenylylate trimer cores and their 5'-monophosphates was also measured in an rRNA cleavage assay using L929 cell extracts. $A_{Rp}A_{Rp}A$ and $A_{Sp}A_{Rp}A$ activated RNase L to cleave 28S and 18S rRNA to specific cleavage products at 10^{-5} M. However, $A_{Rp}A_{Sp}A$ and $A_{Sp}A_{Sp}A$ did not activate RNase L at concentrations as high as 10^{-4} M. It appears that the rRNA cleavage assay was not sensitive enough to detect activation of RNase L by $A_{Rp}A_{Sp}A$. Under the experimental conditions used, authentic A_3 core was also inactive, which is in agreement with previous reports (Haugh et al, *Eur. J. Biochem.* 132:77-84 (1983)).

[0071] The corresponding 5'-monophosphates $PA_{Rp}A_{Rp}A$, $pA_{Sp}A_{Rp}A$ (at 10^{-8} M) and $pA_{Rp}A_{Sp}A$ (at 10^{-7} M) activated RNase L to cleave 28S and 18S rRNA. Authentic pA_3 was active at 10^{-6} M. Incubation with $pA_{Sp}A_{Sp}A$, even at concentrations as high as 10^{-5} M, did not result in detectable rRNA degradation.

[0072] The increased bonding strength of the 5'-phosphorylated trimer core $A_{Rp}A_{Sp}A$ provides a relatively metabolically stable and highly efficient activator for RNase L.

35 [0073] $A_{Sp}A_{Sp}A$ and corresponding 5'-monophosphate were observed to inhibit RNase L activation in both the core-cellulose and rRNA cleavage assays. Notwithstanding, these compounds are extremely useful as probes in the evaluation of the role of RNase L in the interferon-induced biological cascade. Most importantly, $pA_{Sp}A_{Sp}A$ selectively inhibits activation of RNase L at physiological concentrations, and is metabolically stable to specific and non-specific phosphodiesterases. The molecule provides the means to selectively shut off RNase L activation.

40 [0074] Individuals afflicted with chronic myelogenous leukemia ("CML") display a highly elevated RNase L activity, as evidenced by novel rRNA CML-specific cleavage products. Thus, $pA_{Sp}A_{Sp}A$, which is a metabolically stable inhibitor of RNase L, has potential utility in treating myelogenous leukemia.

[0075] $pA_{Sp}A_{Sp}A$ is the most effective inhibitor of RNase L reported to date. Moreover, notwithstanding its RNase L inhibitory effect, $pA_{Sp}A_{Sp}A$ is observed to inhibit HIV reverse transcriptase activity and tobacco mosaic virus replication.

45 [0076] For pharmaceutical use, the compounds of the invention may be taken up in pharmaceutically acceptable carriers, such as, solutions, suspensions, tablets, capsules, ointments, elixirs and injectable composition and the like. They are administered to subjects suffering from viral infection. The dosage administered depends upon the nature and severity of the infection, the disease stage, and, when administered systematically, the size and weight of the infected subject.

50 [0077] The compounds are generally administered in the form of water-soluble salts. Pharmaceutically acceptable water soluble salts include, for example, the sodium, potassium or ammonium salts of the active compounds. They are readily dissolved in water or saline solution. Thus, the preferred formulation for pharmacological use comprises a saline solution of the desired compound in salt form. The formulation may further contain an agent, such as a sugar or protein, to maintain osmotic balance. The salt form of the compound is preferred owing to the relatively high acidity (about pH 3) of the acid form of the compounds.

55 [0078] The compounds of the invention may be used to treat or protect humans and animals from viral infectives such as Herpes simplex, rhinovirus, Epstein Barr virus, measles virus, multiple sclerosis (which may be caused by a viral agent) and the various Human Immunodeficiency Viruses ("HIV") such as HIV-1, which causes cutaneous T cell lymphoma, HIV-2, which causes Sezary lymphoma, and HIV-3, which is responsible for acquired immune deficiency syn-

drome ("AIDS"). The compounds of the invention inhibit the reverse transcriptase activity of HIV.

[0079] The compounds may be applied topically to treat skin cancers caused by radiation, carcinogens or viral agents. Such skin cancers include cutaneous T-cell lymphoma, Sezary lymphoma, Xeroderma pigmentosum, ataxia telangiectasia and Bloom's syndrome. A sufficient amount of a preparation containing a compound of the invention is applied to cover the lesion or affected area. An effective concentration of active agent is between about 10^{-3} M and 10^{-5} M, with 10^{-4} M being preferred.

Effect of 2',5'-Phosphorothioate Oligoadenyates on HIV Reverse Transcriptase Activity

[0080] HIV reverse transcriptase (RNA-dependent DNA nucleotidyl-transferase) activity was assayed by a modification of the procedure of Poiesz et al. (Poiesz, B.J., Ruscetti, F.W., Gazdar, A.F., Bunn, P.S., Minna, J.D., and Gallo, R.C., *Proc. Natl. Acad. Sci. U.S.A.* 77:, 7415-7419 (1980)).

[0081] Cultured H-9 cells are grown at 10^6 cells/ml in RPMI-1640 medium and 20% heat-inactivated fetal calf serum. Cell suspensions are centrifuged (1000 x g, 10 min.) and the supernatant is removed. Virus particles are precipitated from this cell-free supernatant to which 0.3 ml of 4 M NaCl and 3.6 ml of 30% (weight/volume) polyethylene glycol are added. The suspension is placed on ice for 2 hr following centrifugation at 15,000 x g for 30 min at 0°C. The precipitate is resuspended in 200 microliters of 50% glycerol (vol./vol.)/25 mM Tris-HCl (pH 7.5)/5 mM dithiothreitol/50 mM KCl/0.025% Triton X-100. Virus particles are lysed by the addition of 100 microliters of 0.9% Triton X-100/1.5 M KCl. Reverse transcriptase assays are performed at 37°C for 1 hr with 10 microliters of the lysed virus solution in a final reaction volume of 100 microliters containing: 40 mM Tris-HCl (pH 7.8), 4 mM dithiothreitol, 45 mM KCl and 2.5 micrograms of template primer [poly(A)-dT₁₅, 0.5 micrograms/microliter] (with a final Mg⁺⁺ concentration of 10 mM). At this time, 10 microliters of the 2',5'-phosphorothioate oligoadenyate is added to a final concentration of 200 micromolar. Reaction mixtures also contain 4 micromoles of [³H]dTTP. Reactions are stopped by the addition of cold 5% trichloroacetic acid and filtered through nitrocellulose discs. The discs are dried and the radioactivity bound to the discs is determined. Reverse transcriptase activity is expressed as the percent relative to a control.

[0082] The data is shown in Table 5. The compounds were assayed in the above manner in two sets, each set having a separate set of controls. The compounds of reactions 1-4 were assayed against control #1, which was 187×10^3 cpm. The compounds used in reactions 5-13 were assayed against control #2, which was 273×10^3 cpm.

TABLE 5

Inhibition of HIV (HTLV-III _{BH-9}) Reverse Transcriptase Activity by 2',5'-Phosphorothioate Oligoadenyates				
Reaction No.	Compound	Concentration (uM)	Reverse Transcriptase Activity (cpm x 10 ⁻³)	Percent Inhibition
1	p ₃ A ₃	200	187	0
2	pA ₃	200	133	29
3	A ₃	200	201	0
4	pA _{Rp} A _{Rp} A	200	144	25
5	pA _{Sp} A _{Rp} A	200	230	16
6	PA _{Rp} A _{Sp} A	200	206	25
7	PA _{Sp} A _{Sp} A	200	232	15
8	A _{Rp} A _{Rp} A	200	224	18
9	A _{Sp} A _{Rp} A	200	258	6
10	A _{Rp} A _{Sp} A	200	247	10
11	A _{Sp} A _{Sp} A	200	219	20
12	A _{Sp} A _{Sp} A _{Sp} A	200	149	45
13	A _{Rp} A _{Sp} A _{Sp} A	200	147	46

[0083] Although p₃A₃, pA₃ and A₃ are found in mammalian cells, only the monophosphate inhibits HIV reverse transcriptase. The core compounds of the present invention on the other hand are observed to inhibit HIV reverse transcriptase. While all the compounds of the invention (reaction nos. 5,6,7,9,10,11,12 and 13) inhibit the transcriptase to

some degree, the tetramers are particularly effective.

[0084] The compounds of the invention may be administered in amounts of from about 10 micromoles to about 200 micromoles to inhibit HIV reverse transcriptase and treat HIV.

[0085] The compounds also possess antiviral activity against plant-infecting virus, particularly tobacco mosaic virus. Similar results may be obtained against other viruses which cause necrosis in turnips, cucumber, orchids and in other plants. Such viruses include, but are not limited to, tobacco vein mottling virus, vesicular stomatitis virus, vaccinia virus, turnip necrosis virus, and cymbidium orchid virus.

[0086] The compounds may be administered effectively to plants by topical application by abrasion of the leaf surface, aerosol spray, treatment of the soil, spraying, or dusting.

[0087] An effective antiviral composition may be formed by combining one or more of the compounds of the invention with a suitable carrier material. While the individual stereoisomers are preferred for pharmaceutical use, mixtures of one or more of stereoisomers may be employed in agricultural applications. The active compound may also be administered by spraying insect vectors such as aphids, thrips and whiteflies which carry virus to plants. The dosage administered depends upon the severity of the infection.

[0088] The compounds of the invention may be applied to plant seeds prior to germination to control viruses contained in the germ plasm. The seeds may be soaked in a solution of polyethylene glycol ("PEG") containing one or more of the compounds. PEG brings the seeds to physiological activity and arrest. The relative concentration of active compound to PEG depends upon the type of seed under treatment.

[0089] Plants are effectively treated with an aqueous formulation containing from about 10^{-1} to about 10^{-2} M concentration of active ingredient. The compounds of the invention may be applied at very low concentrations. An effective amount of active ingredient on the plant surface is from about 10^{-8} to about 10^{-12} mole per cm^2 of plant surface area, with about 10^{-10} mole to about 10^{-12} mole per cm^2 being preferred. For the typical tobacco plant of 1,000 cm^2 , 10^{-5} M of compound is effective. At this rate, one pound of active ingredient is sufficient to treat 2×10^8 tobacco plants.

[0090] For agricultural application, the compounds are advantageously administered in the form of water-soluble salts, e.g. ammonium or potassium salts. Sodium salts are generally avoided in treating edible plants.

[0091] The compounds of the invention are readily dissolved in water, particularly at such low concentrations. Aqueous formulations for agricultural use may optionally contain a sticker and/or a UV-stabilizer. Such agents are well-known to those skilled in the art. Fatty acids (1%) are useful as spreader sticker agents. Effective UV-stabilizers include, for example, p-aminobenzoic acid.

Effect of 2',5'-Phosphorothioate Oligoadenylates on Tobacco Mosaic Virus (TMV) Replication in Intact Tobacco Plants

[0092] The effectiveness of the 2',5'-phosphorothioate oligoadenylates against plant virus was demonstrated by infectivity tests on intact *Nicotiana glutinosa* plants as follows.

[0093] Carborundum (400 mesh) was sprinkled lightly onto leaves. Solutions containing 0.2 micrograms per ml of TMV and 2×10^{-5} M 2',5'-phosphorothioate oligoadenylate in phosphate buffer were applied onto half-leaves of *N. glutinosa* with either a gloved finger or with a pipettor. The remaining half-leaves were controls (inoculated with the buffer solution containing TMV, but no active compound). The infection was allowed to proceed 48 h under continuous illumination of about 1500 Lx at which time local virus lesions appeared. Inhibition of TMV replication was calculated as the percent of local lesions produced in 2',5'-phosphorothioate oligoadenylate-treated half-leaves compared to control half-leaves. The data is set forth in Table 6.

TABLE 6

Compound ³	Percent TMV Inhibition ⁴
-	0
A ₃	16
A _{Rp} A _{Rp} A	57
A _{Sp} A _{Sp} A	80
A _{Rp} A _{Sp} A	70
A _{Sp} A _{Rp} A	33
P A _{Rp} A _{Rp} A	75
p A _{Rp} A _{Sp} A	15
p A _{Sp} A _{Rp} A	23
p A _{Sp} A _{Sp} A	40
A _{Sp} A _{Sp} A _{Sp} A	70
A _{Rp} A _{Sp} A _{Sp} A	80

³ All compounds were added at 2×10^{-5} M in the infecting solution.

⁴ Inhibition of TMV replication was detected 48 hr after infection and was calculated as the percent of local lesions produced in treated half-leaves of *N. glutinosa* compared to control half-leaves.

[0094] Non-infected plants were treated with 2×10^{-6} M and 2×10^{-5} M of the 2',5'-phosphorothioate core and 5'-monophosphate analogues. No toxicity (chlorosis or necrosis) was observed during the two week period tested.

[0095] Of the ten phosphorothioate trimer and tetramer cores and 5'-monophosphates tested, the SpSp phosphorothioate trimer core inhibited TMV replication to the greatest extent (80%). Similarly, the corresponding tetramer core, SpSpSp, inhibited TMV replication by 70%. The RpSpSp tetramer core inhibited TMV replication by 80%. The remarkable inhibition by A_{Sp}A_{Sp}A, A_{Sp}A_{Sp}A_{Sp}A and A_{Rp}A_{Sp}A_{Sp}A, as well as the 70% inhibition by A_{Rp}A_{Sp}A, compares with only a 16% inhibition with authentic A₃. By introducing the property of chirality, as well as increased metabolic stability, there is a marked increase of the inhibition of TMV replication by the compounds of the invention over 2-5A.

[0096] In addition to administration with conventional carriers, the compounds of the present invention may be administered by a variety of specialized oligonucleotide or nucleic acid delivery techniques. 2-5A and its analogues have been successfully encapsulated in unilamellar liposomes and delivered with the aid of monoclonal antibodies to cells (Bayard et al, *Eur. J. Biochem.* 151:319-325 (1985)). Reconstituted Sendai virus envelopes have been successfully used to deliver RNA and DNA to cells (Arad et al, *Biochem. Biophys. Acta.* 859:88-94 (1986)). These techniques may be utilized for introduction of the present 2',5'-phosphorothioate oligoadenylates into cells.

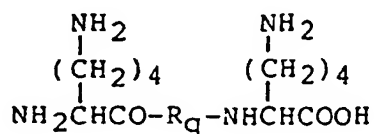
[0097] It is further contemplated that the compounds of the invention may be administered in the form of prodrugs in which lipophilic groups are attached to, for example, the 5'-terminal hydroxyl group of the core compound.

Conjugation of 2',5'-Phosphorothioate Tetramer Adenylates

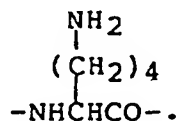
[0098] Poly(L-lysine) has been described as a versatile membrane carrier for 2-5A and other macromolecules (Bayard et al, *Biochem.* 25:3730-3726 (1986)). The tetramer cores and phosphorylated tetramers of the present invention may be conveniently administered in the form of poly(L-lysine) conjugates. The conjugates are formed by introducing two aldehyde functions at the 2' end of the tetramer by periodate oxidation of the alpha-glycol group of the ribose residue. The resulting aldehyde groups are then randomly coupled to the epsilon-amino groups of lysine residues of poly(L-lysine) by Schiff base formation, and then reduced with sodium cyanoborohydride at pH 8.0. This procedure converts the 2',3'-terminal ribose ring into a morpholine structure. The poly(L-lysine) peptide preferably contains from about 60 to about 70 lysine residues. From about five to about ten of the lysine residues are coupled in this manner to tetramer moieties. The resulting 2',5'-phosphorothioate/poly(L-lysine) conjugates may then be isolated by gel filtration chroma-

tography on a Sephadex G-50 column.

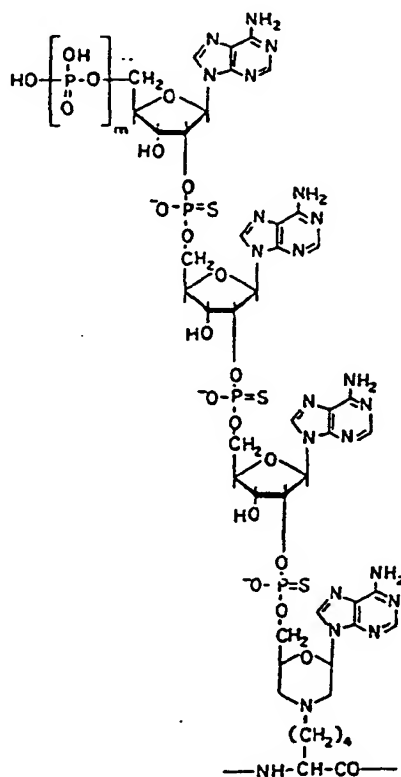
[0099] The poly(L-lysine)/2',5'-phosphorothioate oligoadenylate conjugates have the formula:



wherein q is an integer from about 60 to about 70 and R is randomly R' or



From about five to about ten of the R groups comprise R'. The R' group has the following formula wherein m is 0, 1, 2 or 3:



[0100] The conjugates may be advantageously prepared by the procedure of Bayard et al, **Biochem.**25:3730-3736 (1986):

Example 18**Preparation of Poly(L-Lysine)/2',5'-Phosphorothioate Oligoadenylate Conjugates**

- 5 [0101] A 4-microliter aliquot of sodium metaperiodate (0.6 micromole in 0.1 M sodium acetate buffer, pH 4.75) is added to an ice-cold solution of 2',5'-phosphorothioate tetramer adenylate in 400 microliter of distiller water. The reaction mixture is stirred on ice for 30 min; 400 microliter of poly(L-lysine) (0.14 micromole in 0.2 M phosphate buffer, pH 8.0) and 200 microliter of sodium cyanoborohydride (20 micromole in 0.2 M phosphate buffer, pH 8.0) are added. The mixture is incubated for 2 h at room temperature and then loaded on a Sephadex G-50 column equilibrated with 0.1 M sodium acetate buffer, pH 4.75. Each fraction is assayed for its phosphorothioate oligoadenylate/poly(L-lysine) content by the method described by Lowry et al, *J. Biol. Chem.* 193:265-275 (1951), and by absorbance at 260 nm.
- 10 [0102] Conjugation of the 2',5'-phosphorothioate tetramer to poly(L-lysine) leaves the remaining three 2',5'-linked phosphorothioate adenylic residues intact for optimal RNase L binding and activation.

15 **Liposome Encapsulation of 2',5'-Phosphorothioate Oligoadenylates**

[0103] Encapsulation of the compounds of the present invention comprises another attractive non-disruptive technique for introduction into cells. Liposome encapsulation may be advantageously accomplished according to the technique described by Kondorosi et al., *FEBS Lett.* 120:37-40 (1980):

20

EXAMPLE 19**Preparation of Large Unilamellar Vesicles (Liposomes) Loaded with 2',5'-Phosphorothioate Oligoadenylates**

- 25 [0104] Briefly, a phospholipid mixture from bovine brain (Sigma Chemical Co., Folch fraction III composed of 80-85% phosphatidylserine with the remaining 15% composed of other brain lipids; 35 mg) is suspended in 5 ml of buffer A [0.1 M NaCl, 2 mM histidine, 2 mM N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid ("TES"), 0.4 mM EDTA (pH 7.4) by vortexing. The suspension is sonicated under nitrogen for 10 minutes at 0°C. The suspension is further incubated for 1 hr at 37°C after adjusting the final concentration of Ca⁺⁺ to 20 mM by the addition of 125 microliters of 800 mM CaCl₂. The resulting precipitate is sedimented by centrifugation (2500 x g, 10 min), vortexing and mixing with 100 microliters of 1 x 10⁻⁴ M 2',5'-phosphorothioate oligoadenylate, which is dissolved in phosphate-buffered saline. The final concentration of EDTA is then adjusted to 120 mM by the addition of 400 microliters of buffer B [150 mM EDTA, pH 7.4, 0.1 M NaCl, 2 mM histidine, 2 mM TES]. Liposomes are formed after incubation of this mixture for 30 minutes at 37°C. The excess of EDTA and non-encapsulated components are removed by passing the liposomes through a
- 30 Sephadex G-25 column which is equilibrated with phosphate-buffered saline. About 10% of the 2',5'-phosphorothioate oligoadenylate is encapsulated into liposomes by this procedure. The liposome suspension is stable at 4°C for one week following preparation.

35 **Preparation of Reconstituted Sendai Virus Envelopes Containing 2',5'-Phosphorothioate Oligoadenylates**

- 40 [0105] Reconstituted Sendai virus envelopes may be used as efficient vehicles for the introduction of polynucleotides into cells. Arad et al, *Biochimica et Biophysica Acta* 859:88-94 (1986) disclose introduction of poly(I) • poly(C) into cultured cells by the use of reconstituted Sendai virus envelopes. Fusion of thus-loaded reconstituted Sendai virus envelopes leads to introduction of the enclosed macromolecules into the recipient cell cytoplasm.

- 45 [0106] Reconstituted Sendai virus envelopes may be obtained by detergent solubilization of intact Sendai virus particles. The reconstituted envelopes are fusogenic vesicles consisting of the viral envelope phospholipids and their glycoproteins, devoid of the viral genomic RNA.

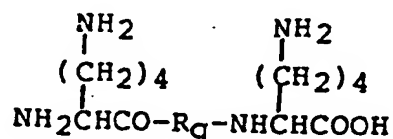
- [0107] Incorporation of the compounds of the present invention into reconstituted Sendai virus envelopes for fusion-mediated micro-injection may be accomplished by following the procedure of Arad et al. Briefly, a pellet of Sendai virus particles (1.5 mg protein) is dissolved in 30 microliters of a solution containing 10% Triton X-100, 100 mM NaCl, 50 mM Tris-HCl (pH 7.4) and 0.1 mM phenylmethylsulfonyl fluoride (Triton X-100:protein ratio, 2:1, w/w). To the clear supernatant obtained after centrifugation, 2',5'-phosphorothioate oligoadenylate dissolved in a solution A (160 mM NaCl, 20 mM Tris-HCl, (pH 7.4)) is added to give a final concentration of active ingredient of 5-20 mg/ml and a final volume of 150 microliters. Triton X-100 is removed from the supernatant by direct addition of 40 mg of SM-2 Bio-Beads. The turbid suspension obtained (containing reconstituted Sendai virus envelopes) is centrifuged at 100,000 x g for 1 h. The pellet, containing about 10% of the original viral protein, is then suspended in solution A to give a final protein concentration of 25 micrograms/ml.
- 50
- 55

[0108] The present invention may be embodied in other specific forms without departing from the spirit or essential

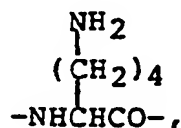
attributes thereof and, accordingly, reference should be made to the appended claims, rather than to the foregoing specification, as indicating the scope of the invention.

Claims

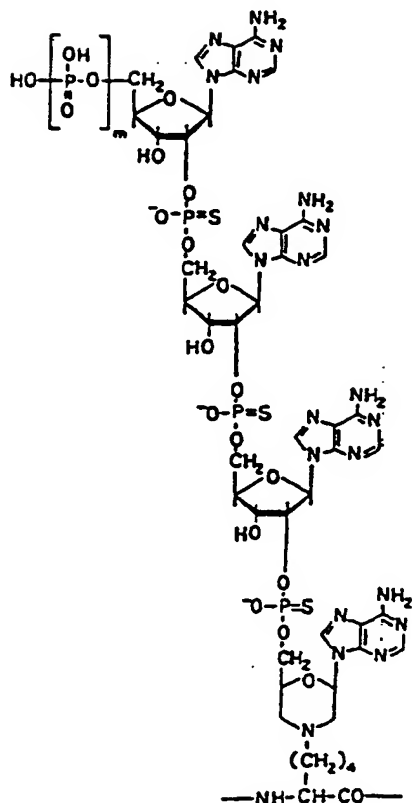
1. A method of controlling viral infection in plants comprising administering an antiviral effective amount of the compound (Rp)-P-thioadenylyl-(2'-5')-(Rp)-P-thioadenylyl-(2'-5')- adenosine, the 5'-mono, 5'-di or 5'-triphosphate thereof, or a water-soluble salt of any such compound.
2. A conjugate of poly(L-lysine) and a 2'-5'-phosphorothioate oligoadenylate, said conjugate having the formula



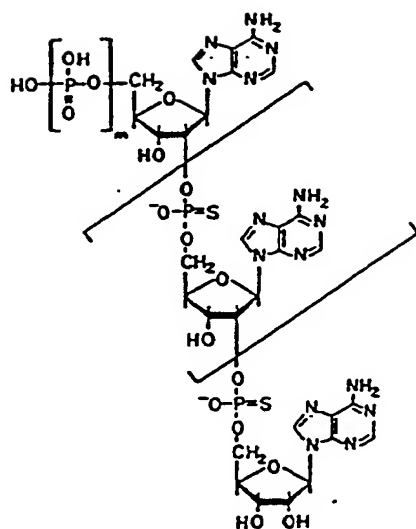
wherein q is an integer from 60 to 70 and R is randomly R' or



and from five to ten of the R groups are R', which R' has the following formula wherein m is 0, 1, 2 or 3:



3. A compound of the formula

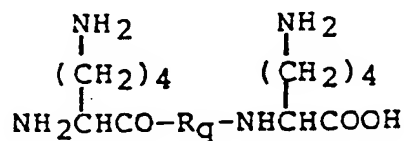


wherein m is zero, 1, 2 or 3 and n is 2, and water-soluble salts thereof.

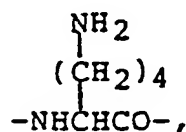
Patentansprüche

1. Verfahren zum Bekämpfen einer Virusinfektion in Pflanzen, welches das Verabreichen einer antiviral wirksamen Menge der Verbindung (Rp)-P-Thioadenylyl-(2'-5'-(Rp)-P-thioadenylyl-(2'-5')-adenosin, deren 5'-Mono-, 5'-Di- oder 5'-Triphosphat oder eines wasserlöslichen Salzes einer derartigen Verbindung umfaßt.

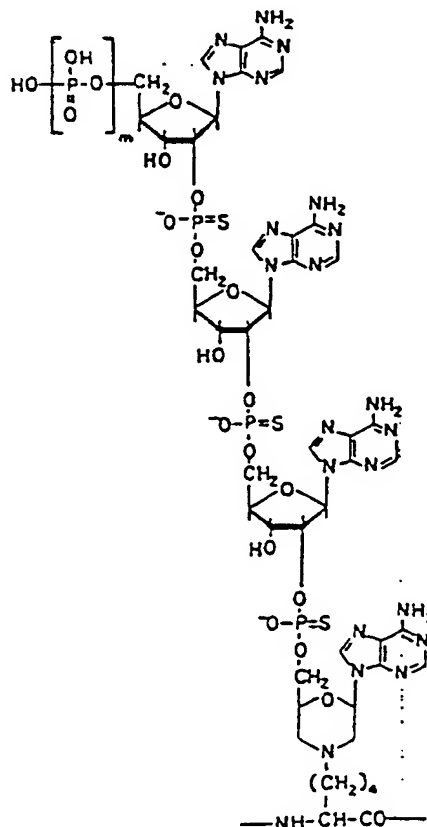
2. Konjugat aus Poly(L-lysin) und einem 2'-5'-Phosphorothioatoligoadenylat, wobei das Konjugat die Formel



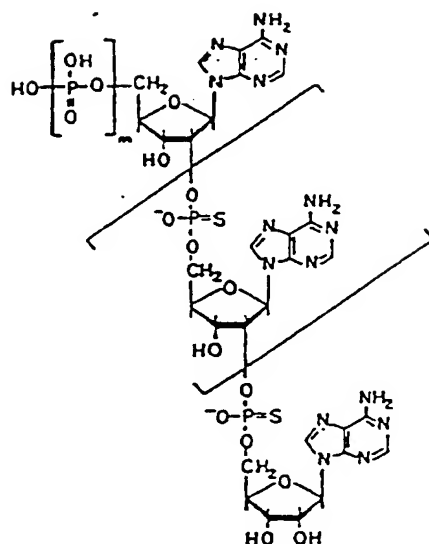
aufweist, worin q eine ganze Zahl von 60 bis 70 ist und R willkürlich ausgewähltes R' oder



ist und fünf bis zehn Gruppen R R' sind, wobei R' die folgende Formel aufweist, in der m 0, 1, 2 oder 3 ist:



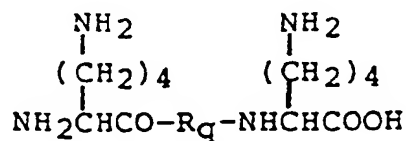
3. Verbindung der Formel



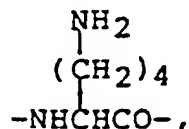
worin m null, 1, 2 oder 3 ist und n 2 ist, und wasserlösliche Salze davon.

Revendications

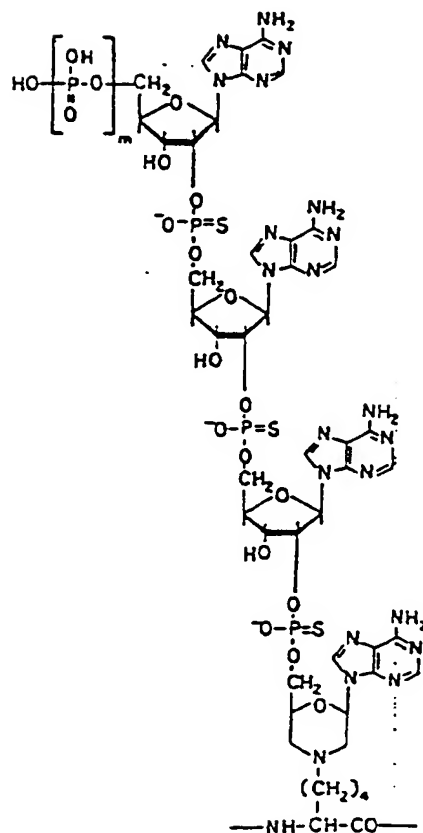
1. Procédé de contrôle de l'infection virale dans les plantes, qui comprend l'administration d'une quantité efficace comme antiviral, du composé (Rp)-P-thioadénylyl-(2'-5')-(Rp)-P-thioadénylyl-(2'-5')-adénosine, le 5'-mono, le 5'-di- ou le 5'-triphosphate de celui-ci ou un sel soluble dans l'eau de n'importe quel de ces composés.
2. Un conjugué de poly(L-lysine) et d'un 2'-5'-phosphorothionate oligoadénylate, ce conjugué ayant la formule :



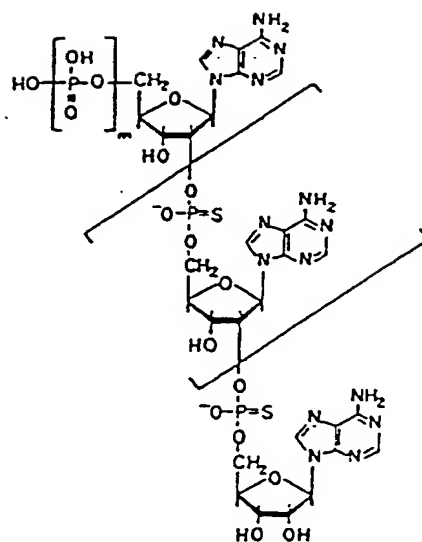
dans laquelle q est un nombre entier allant de 60 à 70 et R est au hasard R' ou :



dans laquelle de 5 à 10 groupes R sont R', lequel R' a la formule suivante où m est 0, 1, 2 ou 3 :



3. Un composé de formule :



dans laquelle m est zéro, 1, 2 ou 3, et n est 2, ainsi que ses sels solubles dans l'eau.

